

Dissection of GnRH Receptor-G Protein Coupling

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Declaration

I hereby declare that the work presented within this thesis was carried out by myself during the course of my PhD and that it has not been submitted for any other degree or qualification. Where I have used the work of others, the sources of information have been detailed clearly in the presentation.

Colin D. White.

Acknowledgements

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Everyone involved in scientific research knows the job has some very good points and some not so good ones. I've been exposed to plenty of both during my time with the MRC. Looking back, I've never been more certain that the pros outweigh the cons. With that in mind, I'd like to thank both of my examiners for allowing me to continue in a career that I absolutely love.

Publications and Presentations

Most of the work presented within chapter three has been published in a paper in *Molecular Endocrinology* (White et al., 2008a). Additionally, I have contributed to a publication in the *Journal of Biological Chemistry* (Lu et al., 2007), authored a review article in *Neuroendocrinology* (White et al., 2008b) and am currently preparing a manuscript to be submitted to the *Journal of Biological Chemistry*. For copies of the published papers, the reader is directed to appendix 7.2. The details of the manuscript in preparation are shown below.

White, CD, Millar, RP and Lu, ZL (2009). GnRH receptor activation of the G_{12/13} G proteins. *J Biol Chem*, Manuscript in Preparation.

Much of the work described throughout this thesis has also been presented at two annual meetings of the Biochemical Society and two annual meetings of the Endocrine Society.

Abbreviations

7-TM	Seven-transmembrane
α GSU	α glycoprotein subunit
AGS	Receptor-independent activator of G protein signalling
AP1	Activator protein 1
ATP	Adenosine-5-triphosphate
BSA	Bovine serum albumin
C3	<i>C. botulinum</i> C3 transferase
cAMP	Cyclic adenosine-5-monophosphate
CDK	Cyclin-dependent kinase
cDNA	Complimentary DNA
CRE	cAMP response element
CTX	Cholera toxin
DAG	Diacylglycerol
DH	Dbl homology
DMEM	Dulbeccos Modified Eagles medium
DMSO	Dimethylsulphoxide
DOI	1-2,5-dimethoxy-4-iodophenyl-2-aminopropane
ECF	Enzyme linked chemifluorescence
ECL	Extracellular loop
EGF	Epidermal growth factor
EGR1	Early growth response 1
EPAC	Exchange protein activated directly by cAMP
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate complex
ERK	Extracellular signal regulated kinase
ETCM	Extended TCM
FAK	Focal adhesion kinase
FRET	Fluorescence resonance energy transfer
FSH	Follicle-stimulating hormone
G	GTP binding
GAP	GTPase activating protein

GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GRK	GPCR kinase
GTP	Guanosine-5-triphosphate
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HH	Hypogonadotropic hypogonadism
IAP	Islet activating protein
IBMX	3-isobutyl-1-methylxanthine
ICL	Intracellular loop
IP ₃	Inositol-1,4,5-trisphosphate
JNK	Jun-N-terminal kinase
LARG	Leukaemia-associated RhoGEF
LB	Luria broth
LSB	Laemmli sample buffer
LSD	Lysergic acid diethylamide
LiSS	Ligand-induced selective signalling
LH	Luteinising hormone
LHX3	Lim homeodomain transcription factor 3
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblast
MOI	Multiplicity of infection
NFY	Nuclear factor Y
PA	Phosphatidic acid
PAK1	p21 activated kinase 1
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PFU	Plaque forming units
PH	Pleckstrin homology
PhI	Phosphoinositide

PI3 kinase	Phosphatidylinositol-3-kinase
PITX1	Pituitary homeobox 1
PKA	Protein kinase A
PKC	Protein kinase C
PLA ₂	Phospholipase A ₂
PLC β	Phospholipase C β
PLD	Phospholipase D
PMT	<i>Pasteurella multocida</i> toxin
PTP	Phosphotyrosine phosphatase
PTX	Pertussis toxin
PVDF	Polyvinylidene difluoride
Pyk2	Pro rich Tyr kinase 2
RGS	Regulator of G protein signalling
Rho	Ras homologue
RNA	Ribonucleic acid
ROCK	Rho kinase
RTK	Receptor Tyr kinase
S1P	Sphingosine-1-phosphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Standard error of the mean
SF1	Steroidogenic factor 1
SFK	Src family of protein Tyr kinases
SH1	Src homology 1
SH2	Src homology 2
SH3	Src homology 3
SHP	SH2 domain containing Tyr phosphatase
SRB	Sulforhodamine B
SRE	Serum response element
STAT3	Signal transducer and activator of transcription 3
TCM	Ternary complex model
TGN	<i>Trans</i> -Golgi network
TM	Transmembrane region

TUNEL	Terminal deoxynucleotidyltransferase-mediated nick end labelling
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Abstract

Hypothalamic gonadotropin-releasing hormone (GnRH) (GnRH I) is the central regulator of the mammalian reproductive system. Most vertebrates studied also possess a second form of GnRH, GnRH II. GnRH I acts on its cognate G protein-coupled receptor (GPCR) on pituitary gonadotropes and activates $G_{q/11}$ -mediated signalling pathways to stimulate the biosynthesis and the release of luteinising hormone (LH) and follicle-stimulating hormone (FSH). Both GnRHs have also been suggested to inhibit cellular proliferation, an action which has largely been proposed to be mediated by the coupling of the receptor to $G_{i/o}$. However, the range of G proteins activated by the GnRH receptor and the signalling cascades involved in inducing antiproliferation remain controversial.

To delineate the G protein coupling selectivity of the mammalian GnRH receptor and to identify the signalling pathways involved in GnRH I-mediated cell growth inhibition, I examined the ability of the receptor to interact with $G_{q/11}$, $G_{i/o}$ and G_s in $G_{\alpha_{q/11}}$ knockout MEF cells. My results indicate that the receptor is unable to interact with $G_{i/o}$ but can signal through $G_{q/11}$. Additionally, my data do not support the suggestion of GnRH receptor- G_s interaction. Furthermore, I show that the GnRH I-induced inhibition of cell growth is dependent on $G_{q/11}$, src and extracellular signal regulated kinase (ERK) but is independent of the activity of protein kinase C (PKC), Ca^{2+} , jun-N-terminal kinase (JNK) or P38. Based on these findings and previous research within our group, I propose a mechanism whereby GnRH I may induce antiproliferation.

Previous studies from our laboratory suggest that the GnRH receptor can adopt distinct active conformations in response to the binding of GnRH I and GnRH II. These data thus account for our hypothesis of ligand-induced selective signalling (LiSS). Given my previous results, I examined the ability of the GnRH receptor to couple to $G_{12/13}$. My work indicates that the receptor can directly activate $G_{12/13}$ and the downstream signalling cascades associated with this G protein family. Indeed, I provide evidence, in several cellular backgrounds, to suggest that GnRH receptor- $G_{12/13}$ -mediated signalling is involved in the regulation of GnRH-induced MAPK

activity, SRE-driven gene transcription and cytoskeletal reorganisation. Furthermore, I propose a role for these G proteins in the transcriptional regulation of LH β and FSH β . Finally, I confirm previous results from our laboratory indicating that the GnRH receptor may interact with src Tyr kinase and show that GnRH I but not GnRH II may, independently of G_{q/11}, stimulate the Tyr phosphorylation and thus the activation of this protein. I propose that this differential signalling accounts for the distinct effects of GnRH I and GnRH II on cellular morphology and SRE-promoted transcriptional activity.

The research presented within this thesis provides evidence to refute published conclusions based on largely circumstantial experimental data, describes novel GnRH receptor signalling pathways and offers support for the concept of LiSS. It may assist in the development of new therapeutic compounds which selectively target one GnRH-mediated signalling pathway while bypassing others.

Chapter One

Literature Review

1.0 Introduction

This literature review is divided into three parts. In the first, guanosine-5-triphosphate (GTP) binding (G) protein-coupled receptors (GPCRs) will be introduced and a summary of both G protein-dependent and -independent signalling by GPCRs given. Subsequently, the concept of ligand-induced receptor activation and signalling will be reviewed and, finally, gonadotropin-releasing hormone (GnRH) and the signalling associated with the activation of the GnRH receptor examined.

1.1 GPCRs and G Protein-Dependent and -Independent Signalling

With more than 800 members, the G protein-coupled receptor family represents the largest group of cell surface molecules involved in eukaryotic signal transduction and accounts for approximately 2% of the total genes encoded by the human genome (Fredriksson et al., 2003). Their dysfunction contributes to some of the most prevalent diseases affecting man as represented by the fact that they are targeted by approximately 50% of all therapeutic agents currently in clinical use (Dorsam and Gutkind, 2007, Fredholm et al., 2007). In the following sections, an overview of GPCR structure and classification, synthesis and trafficking, activation and both G protein-dependent and -independent signalling is provided.

1.1.1 GPCR Structure and Classification

In 1986, the cloning of the gene and complementary DNA (cDNA) for the mammalian β_2 -adrenergic receptor, and the realisation that it shares a high degree of sequence homology and a predicted seven-transmembrane (7-TM) structure with bovine rhodopsin, led to the idea that a large family of receptors existed (Dixon et al., 1986). This hypothesis was rapidly confirmed by the successful cloning of the muscarinic acetylcholine receptor (Kubo et al., 1986), the α_2 -adrenergic receptor (Kobilka et al., 1987) and the serotonin 5-HT_{1A} receptor (Fargin et al., 1988). GPCRs are therefore known to share a common structural architecture (figure 1.1). They are composed of a single polypeptide with 7-TM α helices connected by alternating hydrophilic intracellular and extracellular loops (ICLs and ECLs

respectively). The amino-terminus is located on the extracellular side of the membrane and the carboxy-terminus on the intracellular side.

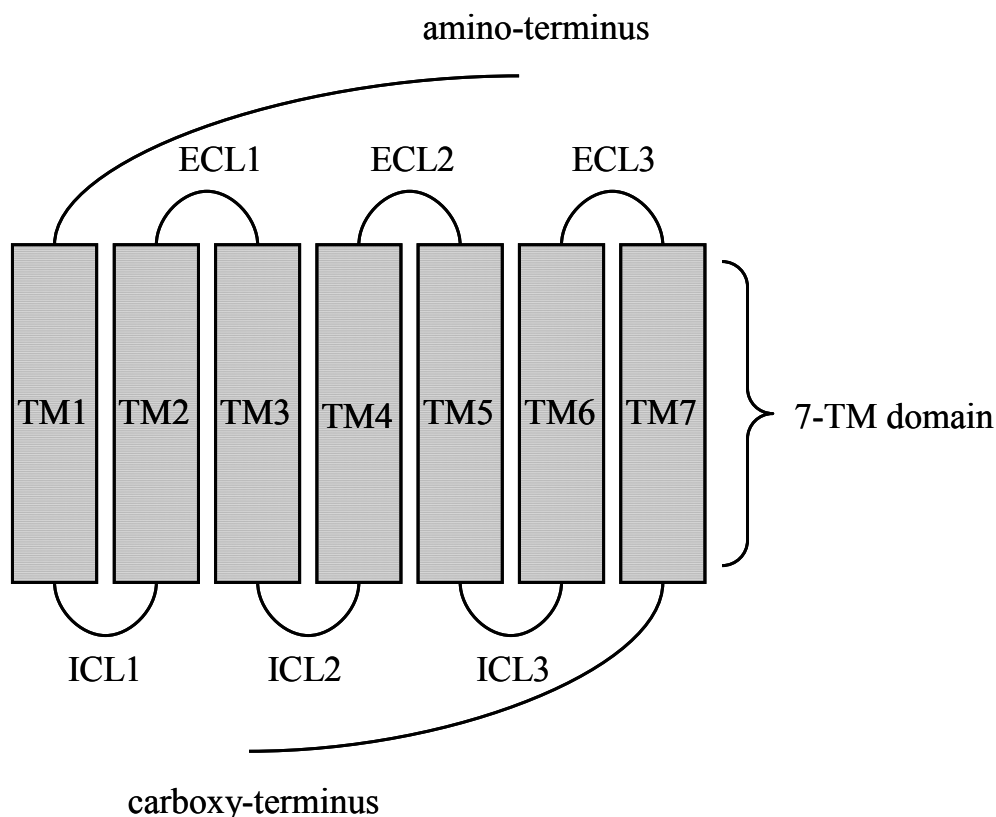


Figure 1.1 Overall topology of the GPCR family. Members of the GPCR family possess a conserved structure consisting of 7-TM α helices (7-TM domain) connected by alternating hydrophilic ICLs and ECLs, an amino-terminus on the extracellular side of the membrane and a carboxy-terminus on the intracellular side.

High resolution structural information is essential for understanding the molecular mechanisms of protein function (Kobilka and Schertler, 2008, Wess et al., 2008). Structural analysis of GPCRs has, however, been somewhat hindered by the inherent difficulties associated with producing and purifying sufficient amounts of recombinant protein (Kobilka, 1995). In 2000, Palczewski and colleagues described the three dimensional crystal structure of bovine rhodopsin at a resolution of 2.8 Å (Palczewski et al., 2000) and thus confirmed the arrangement of the 7-TM domain (Meng and Bourne, 2001, Lu et al., 2002). More recently, crystal structures have been determined for the human β_2 -adrenergic receptor (Cherezov et al., 2007,

Rasmussen et al., 2007, Rosenbaum et al., 2007), the turkey β_1 -adrenergic receptor (Warne et al., 2008) and the human A_{2A} adenosine receptor (Jaakola et al., 2008). These structures highlight the similarities and the differences between different GPCRs and provide improved templates for homology modelling of other receptors. However, crystal structures of GPCRs bound to different ligands and/or in complex with G proteins or other signalling partners are still largely undefined (Scheerer and colleagues very recently published the first crystal structure of opsin in its G protein interacting conformation (Scheerer et al., 2008)). Interestingly, Park and colleagues described the crystal structure of ligand free opsin at 2.9 Å (Park et al., 2008). This represents the first successful crystallisation of the opsin apoprotein in the empty state. The authors postulate that it may provide insight into the retinal uptake and release mechanism as well as ligand binding to, and the activation of, GPCRs in general.

Several classification systems have been adopted to further divide the GPCR family. Attwood and Findlay made the first attempt in 1993 when they devised a system based on the sequence homology of the 7-TM domains (Attwood and Findlay, 1993). Later, in 1994, they extended their dataset from 240 to 393 and noted that 61 sequences made imperfect matches suggesting that motifs within the transmembrane regions are not conserved between GPCR subfamilies (Attwood and Findlay, 1994). In the same year, Kolakowski presented the well known A, B, C, D, E, F and O classification system which was designed to be representative of all GPCRs (Kolakowski, 1994). The usefulness of this system is, however, debated given that some subfamilies do not exist in humans. Fungal pheromone receptors and 3,5-cyclic adenosine-5-monophosphate (cAMP) receptors are represented by subfamilies D and E respectively and subfamily F contains archaebacterial opsins. Following publication of the first draft of the human genome in 2001 (Lander et al., 2001, Venter et al., 2001), Fredriksson and colleagues divided 802 known and predicted human GPCRs into subfamilies based on phylogenetic criteria (Fredriksson et al., 2003). The authors noted that there exist five subfamilies of GPCRs in humans termed rhodopsin, glutamate, secretin, adhesion and frizzled/taste2.

1.1.2 GPCR Synthesis and Trafficking

As with all nascent integral membrane proteins, the life of GPCRs begins in the endoplasmic reticulum (ER) where they are synthesised and folded (Drake et al., 2006, Gurevich and Gurevich, 2008). Correctly folded receptors are packaged into ER derived COP2 coated vesicles (Barlowe et al., 1994) which traverse from the ER to the ER-Golgi intermediate complex (ERGIC), the Golgi apparatus and the *trans*-Golgi network (TGN) (Duvernay et al., 2005). It is during this migratory step that they may undergo post-translational modification (such as glycosylation) before final targeting to the plasma membrane (figure 1.2). Evidence suggests that GPCR export trafficking is a highly regulated process (Dong et al., 2007). Interaction with ER chaperones promotes the proper folding of the immature receptor and stabilises this conformation thereby promoting the delivery to the plasma membrane. Additionally, homo- and hetero-dimerisation of GPCRs may play an important role. Indeed, some studies have indicated that it is a requirement for the successful delivery of certain receptors (such as the α_{1B} -, the α_{1D} - (hetero-dimerisation) and the β_2 -adrenergic (homo-dimerisation) receptors) from the ER to the cell surface (Hague et al., 2004, Salahpour et al., 2004).

Numerous quality control mechanisms ensure that, in most cases, improperly or incompletely folded proteins are targeted for degradation (Bernier et al., 2004). As such, mutations which result in the misfolding and the subsequent misrouting of a GPCR are known to be the cause of a number of human pathologies (Tan et al., 2004, Conn et al., 2007). Examples of such ailments include retinitis pigmentosa (rhodopsin) and hypogonadotropic hypogonadism (HH) (the GnRH receptor). Interestingly, it has been suggested that membrane permeant small molecule GPCR antagonists (which act as pharmacological chaperones) can correct mutant GPCR misfolding and thus facilitate the proper trafficking of the receptor to the plasma membrane (Milligan et al., 2002, Bernier et al., 2004, Conn et al., 2007). At the cell surface, certain mutants (such as E90K with reference to the GnRH receptor) have been shown to be functional at levels comparable with wild-type GPCRs (Morello et al., 2000, Janovick et al., 2002). This pharmacological chaperone action of GPCR

ligands may therefore provide a new approach for the clinical intervention in molecular diseases.

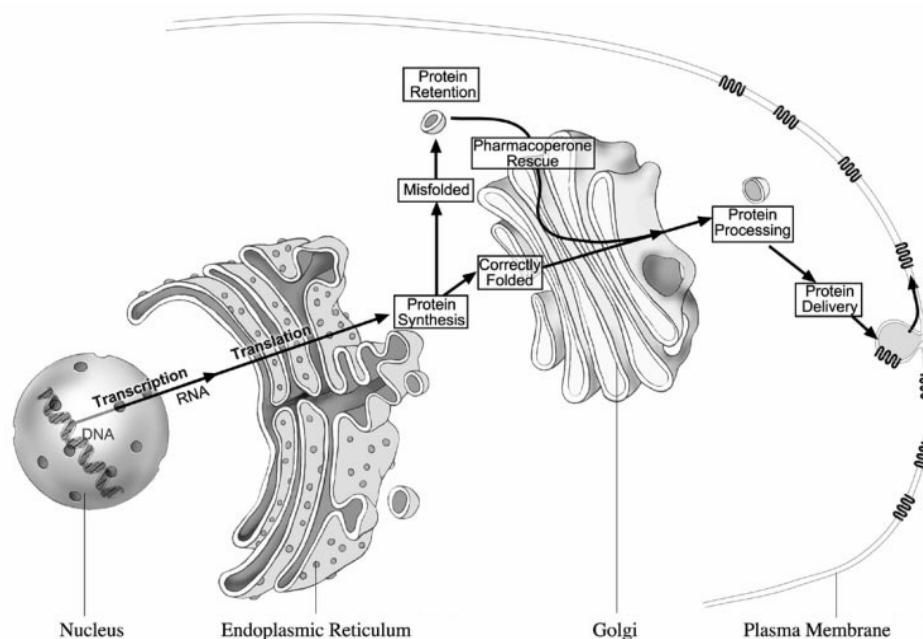


Figure 1.2 GPCR synthesis and export. GPCRs are synthesised and folded in the ER. They migrate from the ER through the Golgi and are finally delivered to the plasma membrane. Misfolded receptors are usually targeted for degradation however membrane permeant GPCR antagonists may be able to rescue receptor surface expression. Figure adapted from Conn et al., 2007. G protein-coupled receptor trafficking in health and disease: lessons learned to prepare for therapeutic mutant rescue *in vivo*. *Pharmacol Rev*, **59**, 225-250.

After the successful delivery to the plasma membrane, binding of an agonist causes the receptor to change conformation leading to the recruitment and the activation of intracellular effectors. Thereafter, receptor desensitisation (figure 1.3) and internalisation may sequentially take place. Initially, GPCR kinases (GRKs) recognise activated GPCRs and become themselves catalytically activated (Premont and Gainetdinov, 2007). This results in the phosphorylation of the receptor at both Ser and Thr residues localised within either ICL3 or the carboxy-terminal tail (Ferguson, 2001). Subsequently, high affinity arrestin binding to the phosphorylated receptor takes place, sterically hindering receptor-G protein interaction (Moore et al., 2007). Additionally, the arrestins serve as adapter molecules linking GPCRs to the

endocytic machinery of the cell (Marchese et al., 2003, Reiter and Lefkowitz, 2006). Interestingly, as well as facilitating receptor internalisation, evidence suggests that the arrestins are able to act as signal transducers in their own right by recruiting a broad spectrum of signalling molecules to GPCRs in a strictly activation-dependent fashion (Lefkowitz and Shenoy, 2005, Lefkowitz, 2007). Following internalisation, GPCRs are subject to one of two sorting fates in the early endosome - recycling or degradation. The recycling pathway facilitates the return of the internalised receptors to the cell surface resulting in functional resensitisation and the degradative pathway results in their transport to lysosomes prior to proteolysis (Hanyaloglu and von Zastrow, 2008).

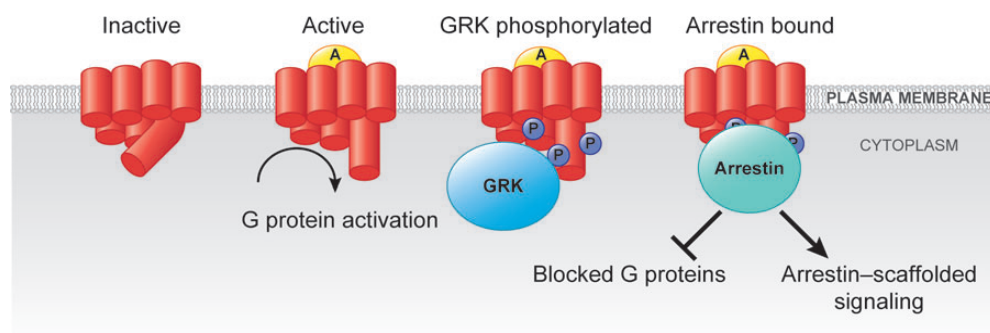


Figure 1.3 GPCR desensitisation. Agonist-induced receptor activation facilitates G protein activation and signalling (Active). The GPCR may then be phosphorylated by GRKs (GRK phosphorylated) prior to arrestin binding (Arrestin-bound). Arrestin binding sterically hinders receptor-G protein interaction, facilitates GPCR internalisation and may initiate a second wave of signalling. Internalised GPCRs are sorted for recycling or degradation. Figure taken from Premont and Gainetdinov, 2007. Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol*, **69**, 511-534.

1.1.3 GPCR Activation

Various theoretical models that explain and predict receptor behaviour have been proposed. In 1980, DeLean and colleagues put forward the ternary complex model (TCM) (DeLean et al., 1980). In this study, experiments on the frog and the turkey erythrocyte β -adrenergic receptor provided a general model for the activation, by agonists, of GPCR systems. The model states that the activation of a GPCR requires the agonist-promoted formation of an active ternary complex (HRX) consisting of agonist (H), receptor (R) and G protein (X). In contrast, the inactive state of a GPCR

is characterised by the absence of the G proteins in the system. This theory is supported by the earlier observation that an agonist-receptor complex ($[^3\text{H}]$ hydroxybenzyl-isoproterenol- β_2 -adrenergic receptor) had an apparent larger molecular size than an antagonist-receptor complex ($[^3\text{H}]$ dihydroalprenolol- β_2 -adrenergic receptor) (Limbird and Lefkowitz, 1978).

In 1993, a study by Samama and colleagues extended the TCM to include the capability of receptors to, in the absence of an agonist, adopt an active conformation and couple to a G protein (Samama et al., 1993). Mutation of Leu²⁶⁶, Lys²⁶⁷, His²⁶⁹ and Leu²⁷² of the β_2 -adrenergic receptor to the corresponding residues from the hamster α_{1B} -adrenergic receptor (Ser²⁶⁶, Arg²⁶⁷, Lys²⁶⁹ and Ala²⁷²) was sufficient to cause the agonist-independent activation of adenylyl cyclase. The extended TCM (ETCM) accommodates both the observation of the ligand-independent receptor-mediated activation of the G proteins and the behaviour of the various classes of ligand. According to the model, GPCRs exist in equilibrium between an inactive state (R) and an active state (R*). They must undergo transition from R into R* in order to bind to and activate the G proteins. In the absence of agonist, the equilibrium is shifted towards the R state. Nevertheless, a proportion of the receptors may spontaneously move into the R* state as the energy barrier between R and R* is sufficiently low. Mutations which result in constitutively active GPCRs alter the equilibrium between R and R* and shift a higher proportion of the receptor molecules into R*. Traditional agonists bind with the highest affinity to the receptors in the R* state, shift the equilibrium towards R* and elicit a functional response. Neutral antagonists bind with the same affinity to the receptors in both the R and the R* states and thus cause no change in the equilibrium between the two. They do, however, prevent the binding of an agonist and therefore avert any cellular response. Inverse agonists stabilise the inactive R state shifting the equilibrium away from R*. Basal receptor activity is inhibited and basal receptor signalling is decreased. The ETCM has been instrumental in shaping our view of the receptor activation process. Specifically, it highlights that agonist-induced receptor-mediated signalling requires the transition of the receptor from an inactive to an active conformation.

Mutational approaches have shed light on the process of GPCR activation by agonist ligands (figure 1.4). For receptors of the rhodopsin subfamily, these studies suggest that small ligands are able to enter deep within the central crevice of the receptor while larger peptide ligands mainly interact with the ECLs and the exofaces of the transmembrane regions (TMs) (Gether, 2000, Lu et al., 2002). Additionally, they infer that, upon agonist binding, TM3 and TM6 play a critical role in the transition of GPCRs to their fully activated conformation. Specifically, an anticlockwise rotation of TM6 (when viewed from the extracellular side), relative to TM3, accompanied by an outward movement of both intracellular ends is thought to be involved in the activation process. Agonist-induced changes in receptor conformation with reference to TM3 and TM6 are supported by a number of elegant studies. Farrens and colleagues utilised Cys spin labelling and disulphide cross linking to demonstrate the involvement of these TMs in rhodopsin activation (Farrens et al., 1996). Magnetic dipolar interactions between the spin labels in TM3 and TM6 revealed their proximity. Furthermore, changes in these interactions upon receptor activation suggested the occurrence of a rigid body movement of these α helices. Indeed, TM3-TM6 disulphide cross linking prevents the rhodopsin-mediated activation of G_t , data which demonstrate the importance of such a movement. Additionally, Sheikh and colleagues engineered Zn^{2+} binding sites between the intracellular surfaces of the transmembrane helices of rhodopsin with the aim of restraining specific activation-induced conformational changes (Sheikh et al., 1996). Mutation of residues in TM3 (V138H) and TM6 (T251H) blocked rhodopsin activation in the presence of Zn^{2+} . Later, the authors reported similar findings at the β_2 -adrenergic receptor and the parathyroid hormone receptor (Sheikh et al., 1999). These data indicate a direct reliance of receptor activation on a change in the spatial arrangement of TM3 and TM6.

In 1999, Elling and colleagues, based on presumed agonist interaction sites on the exofacial regions of TM3 and TM7 of the β_2 -adrenergic receptor, constructed a Zn^{2+} binding site between residues in these TMs (D113H and N312C respectively) (Elling et al., 1999). Signal transduction in the mutant receptor was not activated by β_2 -adrenergic receptor agonists (such as pindolol) but increases in cAMP accumulation

were apparent after the addition of Zn^{2+} . These findings were later replicated with the neurokinin NK_1 receptor (Holst et al., 2000). In this study, the authors concluded that the observed partial agonism in response to Zn^{2+} was due to the lack of critical interactions with residues in TM6. Similarly, Lu and colleagues showed that His substitution of residues in TM3 (L116H), TM6 (F374H) and TM7 (N414H) creates high affinity Zn^{2+} binding sites in the M_1 -muscarinic receptor and thus demonstrated their close proximity in the inactive state (Lu and Hulme, 2000). Ala substitution (L116A), however, had previously been shown to increase the acetylcholine binding affinity and the basal receptor activity implying a shift of the receptor into the active conformation (Lu and Hulme, 1999). Taken together, these studies demonstrate that TM7 also undergoes spatial rearrangement during GPCR activation.

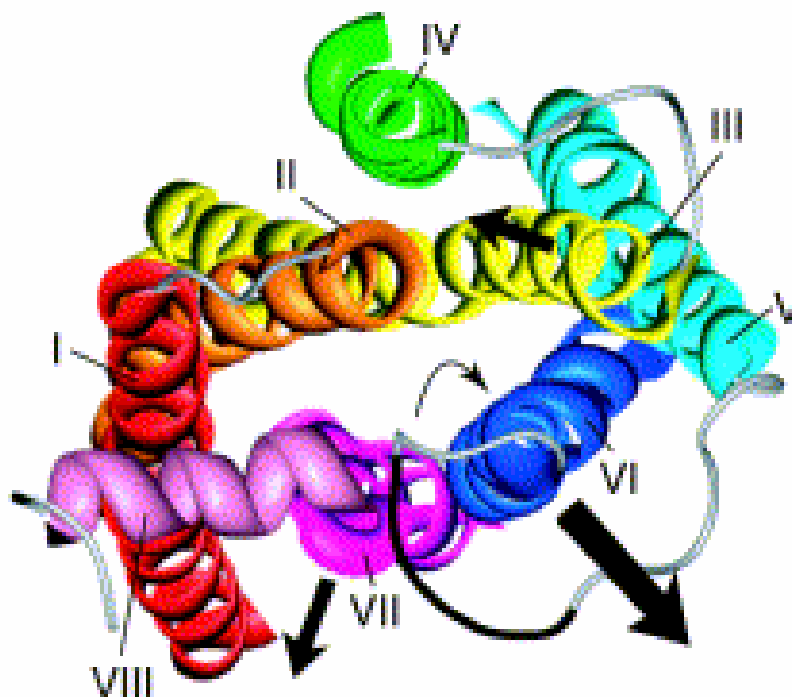


Figure 1.4 Conformational changes during GPCR activation. Upon binding of agonist ligands, rotation of TM6 (VI) (thin arrow), relative to TM3 (III), accompanied by an outward movement of the intracellular ends of TM3, TM6 and TM7 (VII) (thick arrows) occurs. The α helices and the ICLs of rhodopsin are shown as seen from the intracellular side. Figure adapted from Meng and Bourne, 2001. Rhodopsin activation: what does the rhodopsin structure tell us? *Trends Pharmacol Sci*, **22**, 587-593.

1.1.4 GPCR-Mediated G Protein-Dependent Signalling

Despite some structural diversity between the GPCR subfamilies, the G protein round of signalling initiated by these receptors is remarkably uniform (Gurevich and Gurevich, 2008) (figure 1.5). As discussed earlier, when an agonist interacts with a GPCR on the surface of a cell it induces a conformational change of the receptor that activates the heterotrimeric G proteins (composed of $G\alpha$, $G\beta$ and $G\gamma$ subunits) on the intracellular side of the membrane (Cabrera-Vera et al., 2003). Both the $G\alpha$ and the $G\gamma$ subunits are thought to have lipid modifications that serve as hydrophobic membrane anchors (Wedegaertner et al., 1995). In the basal state, the guanosine-5-diphosphate- (GDP) bound $G\alpha$ subunit and the $G\beta\gamma$ complex are associated. Upon interaction of the activated receptor with the heterotrimeric complex, a series of conformational changes are propagated through the $G\alpha$ subunit to the GDP binding site and GDP is exchanged for GTP (McCudden et al., 2005). Subsequently, according to the traditional G protein activation model, the GTP-bound $G\alpha$ subunit dissociates from the $G\beta\gamma$ complex. Both the $G\alpha$ -GTP subunit and the $G\beta\gamma$ complex are now free to modulate the activity of a variety of intracellular effectors. The duration of this modulation is determined by the intrinsic GTPase activity of the $G\alpha$ subunit (although there exist mechanisms that accelerate signal termination). The activation cycle is completed by the hydrolysis of GTP to GDP and the subsequent reassociation of the $G\alpha$ -GDP subunit with the $G\beta\gamma$ complex.

More recent models concerned with G protein activation have suggested that the dissociation of the subunits that make up the heterotrimeric complex may not be absolutely required for downstream signalling (Robishaw and Berlot, 2004). This theory, termed the clamshell model of G protein activation, is supported by fluorescence resonance energy transfer (FRET) studies of fluorescently tagged G protein subunits. Bunemann and colleagues showed that, although a conformational change takes place upon G protein activation, dissociation of the $G\alpha$ subunit ($G\alpha_{i1}$) and the $G\beta\gamma$ complex does not necessarily occur (Bunemann et al., 2003). According to the model, GPCR activation-induced changes in G protein conformation expose previously inaccessible domains on the $G\alpha$ subunit and the $G\beta\gamma$ complex. This conformational change is necessary for the activation of intracellular effectors and

suggests that G proteins may act more as scaffolds for the recruitment of other signalling molecules. Signal termination occurs, not by the reassociation of the heterotrimeric complex, but by another set of conformational changes that obscure the effector interaction sites on the $G\alpha$ subunit and the $G\beta\gamma$ complex.

Although the intrinsic GTPase activity of the $G\alpha$ subunit dictates the duration of G protein-mediated effector modulation, and effector-mediated negative feedback can regulate this activity, this regulation is not exclusive. Instead, it can be augmented by a number of factors. For example, some of the $G\alpha$ subunits (such as $G\alpha_z$ and $G\alpha_{12}$) and a $G\gamma$ subunit ($G\gamma_{12}$) may be modified by phosphorylation and this modification also appears to play a role in the regulation of signalling duration (Chen and Manning, 2001). Indeed, protein kinase C- (PKC) induced phosphorylation of $G\alpha_z$ has been shown to inhibit the interaction of this $G\alpha$ subunit with the regulator of G protein signalling (RGS) proteins (RGS_{Z1}) (Wang et al., 1998). Inhibition of this interaction may therefore prolong the activation of $G\alpha_z$ -mediated signalling. RGS proteins are a large family of signalling proteins which share a conserved signature catalytic domain (RGS domain) (Hollinger and Hepler, 2002). However, many are multifunctional in that they possess two or more domains which can mediate protein-protein interactions (Birnbaumer, 2007). Initially, they were considered to act simply as GTPase activating proteins (GAPs) that bind directly to activated $G\alpha$ subunits (Watson et al., 1996). More recently, however, evidence suggests that more complex RGS proteins (those which possess many domains) may act as integrators rather than negative regulators of G protein signalling. One key example comes from the observation that RGS proteins (specifically, in this case, p115 RhoGEF) can directly link the $G\alpha_{13}$ subunits to the activation of the Rho monomeric G proteins. p115 RhoGEF consists of a RGS domain, a Dbl homology (DH) domain and a pleckstrin homology (PH) domain (figure 1.6). The RGS domain acts as a GAP for $G\alpha_{12}$ and $G\alpha_{13}$ (Kozasa et al., 1998) while the DH domain is required for Rho guanine nucleotide exchange activity (Hart et al., 1998). It is thought that the PH domain is necessary for plasma membrane localisation of the protein (Bhattacharyya and Wedegaertner, 2003).

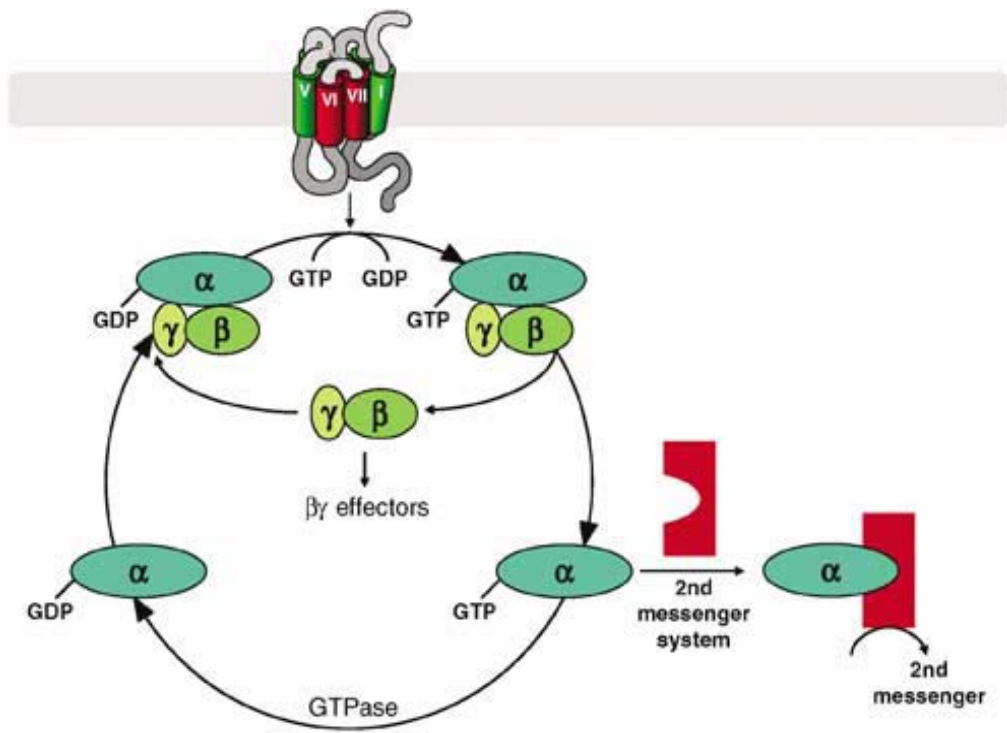


Figure 1.5 GPCR-mediated G protein-dependent signalling. In the inactive state, the GDP-bound $G\alpha$ subunit is associated with the $G\beta\gamma$ complex. GPCR activation induces a conformational change in the $G\alpha$ subunit which results in the exchange of GDP for GTP. This facilitates the dissociation of the GTP-bound $G\alpha$ subunit from the $G\beta\gamma$ complex. Both the GTP-bound $G\alpha$ subunit and the $G\beta\gamma$ complex may go on to modulate the activity of various intracellular effectors. The duration of the cycle is determined by the intrinsic GTPase activity of the $G\alpha$ subunit. The hydrolysis of GTP to GDP and the subsequent reassociation of the heterotrimeric complex completes the cycle. Figure adapted from Milligan and Kostenis, 2006. Heterotrimeric G proteins: a short history. *Br J Pharmacol*, **147**, s46-s55.

p115RhoGEF



Figure 1.6 Structure of p115 RhoGEF. p115 RhoGEF consists of a RGS domain, a DH domain and a PH domain. The RGS domain acts as a GAP for $G\alpha_{12}$ and $G\alpha_{13}$ while the DH domain is required for $G\alpha_{13}$ -mediated Rho guanine nucleotide exchange activity. The PH domain is thought to be necessary for plasma membrane localisation. The protein is orientated such that the amino-terminus is shown on the left and the carboxy-terminus on the right. Figure adapted from Hollinger and Hepler, 2002. Cellular regulation of RGS proteins: modulators and integrators of G protein signalling. *Pharmacol Rev*, **54**, 527-559.

According to current knowledge, sixteen genes encode the $G\alpha$ subunits, five encode the $G\beta$ subunits and twelve encode the $G\gamma$ subunits in man (Cabrera-Vera et al., 2003). Classically, on the basis of the sequence homology of the $G\alpha$ subunit, the heterotrimeric G proteins are divided into four families (G_s , $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) which define, to a large extent, effector specificity (Neves et al., 2002). The exception appears to arise when a signal is promulgated by the $G\beta\gamma$ complex.

Conversion of a G protein heterotrimeric complex from the inactive to the active state is promoted by an interaction with GPCRs. However, another class of signalling proteins, termed the receptor-independent activators of G protein signalling (AGS), has also been identified. As their name suggests, the AGS proteins activate heterotrimeric G protein complexes independently of receptor activation. The $G\alpha$ subunits which they positively regulate, however, appear to differ between members of the protein family. Indeed, Takesono and colleagues demonstrated that, in yeast strains selectively expressing different $G\alpha$ subunits, AGS3 is only efficient in promoting the activation of $G\alpha_{i2}$ and $G\alpha_{i3}$ whereas AGS2 activates $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{16}$ (Takesono et al., 1999). Additionally, recent evidence suggests that the G protein subunits may be activated by other families of receptors. Shan and colleagues showed that $G\alpha_{13}$ is essential for the receptor Tyr kinase- (RTK) induced migration of mouse embryonic fibroblast (MEF) cells to occur (Shan et al., 2006). Furthermore, $G\alpha_{13}$ involvement in cell migration is retained in a carboxy-terminus $G\alpha_{13}$ mutant (which is defective in GPCR coupling) suggesting that the platelet derived growth factor- (PDGF) induced migration is independent of GPCR signalling. Conversely, GPCRs do not exclusively activate the heterotrimeric G proteins. Several lines of evidence indicate that they may also regulate the activity of the monomeric G proteins (Bhattacharya et al., 2004) as well as the non-G protein effectors such as the arrestins (Lefkowitz and Shenoy, 2005) and the src family Tyr kinases (Sun et al., 2007b). These observations add to the already extensive signalling repertoire initiated by GPCRs making them truly one of the most interesting families of signalling proteins currently under investigation. Each of the $G\alpha$ subunits and the $G\beta\gamma$ complex will be discussed in more detail in the following

sections. Additionally, the monomeric G proteins and GPCR-mediated G protein-independent signalling will be reviewed.

1.1.4.1 **G α**

G α subunits contain two distinct domains joined by two linker regions (linker 1 and linker 2) (Lambright et al., 1994). The GTPase domain is conserved throughout the G protein family and the α helical domain is unique to the G α subunits. Between these domains lies a deep cleft within which the guanine nucleotide is tightly bound. Evidence suggests that the α helical domain is divergent between G protein families. As such, this domain has been proposed to represent the G α effector binding site (Masters et al., 1986, Coleman et al., 1994). Additionally, it has been suggested that this region is involved in the interaction of the G α subunits with the RGS proteins (Skiba et al., 1999, Kreutz et al., 2007).

1.1.4.1.1 **G α_s**

The initial demonstration by Sutherland and colleagues (which revealed that cAMP accumulation is stimulated by both glucagon and epinephrine) was the first in a series of milestones that would eventually lead to the successful purification of the G α_s G protein α subunits (Berthet et al., 1957, Rall and Sutherland, 1958, Sutherland and Rall, 1958). It was later realised that there is a GTP-dependent step in the hormonal regulation of cAMP (the ATP used as a previous substrate was contaminated with sufficient GTP to disguise this requirement), data which at the time implied that there existed a stimulatory regulatory component (Rodbell et al., 1971). This was confirmed in a series of landmark papers (Ross and Gilman, 1977, Ross et al., 1978, Northup et al., 1980, Northup et al., 1983) and culminated in Alfred Gilman and Martin Rodbell being awarded the 1994 Nobel Prize for Physiology or Medicine. We now know that it is the ubiquitously expressed G α_s G protein α subunits that mediate GPCR-dependent adenylyl cyclase activation. G α_s can be activated by a number of well characterised GPCRs such as the β_2 -adrenergic receptor, the D₁ dopamine receptor and the H₂ histamine receptor. In turn, membrane-bound adenylyl cyclase isoforms catalyse the conversion of adenosine-5-triphosphate (ATP) to cAMP. In 1968, Walsh and colleagues purified a cAMP-

dependent protein kinase from rabbit skeletal muscle and, in doing so, identified what is now commonly known as protein kinase A (PKA) (Walsh et al., 1968). PKA is a major effector of cAMP signalling and catalyses the phosphorylation of target proteins, such as src Tyr kinase (Schmitt and Stork, 2002), on Ser and Thr residues. In addition, evidence suggests that cAMP may exert PKA-independent effects. DeRooij and colleagues characterised Epac (exchange protein activated directly by cAMP) as a PKA-independent Rap1GEF (DeRooij et al., 1998).

As well as mediating the activity of the adenylyl cyclase isoforms, $G\alpha_s$ is thought to be involved in the direct regulation of other effectors (table 1.1). Ma and colleagues demonstrated that $G\alpha_s$ stimulates the Tyr kinase activity of src and hck by directly binding to their SH1 domains (Ma et al., 2000). The $G\alpha$ subunit was shown to alter their protein conformations facilitating an increase in substrate accessibility to their active sites. Additionally, Roychowdhury and colleagues demonstrated that this $G\alpha$ subunit upregulates the GTPase activity of tubulin and, in doing so, inhibits microtubule assembly (Roychowdhury et al., 1999). This variety of effector regulation, however, is apparently not shared by $G\alpha_{olf}$, another member of the G_s G protein family. $G\alpha_{olf}$ was originally characterised in 1989 as a G protein α subunit specific for olfactory tissue (Jones and Reed, 1989). It was shown to activate adenylyl cyclase isoforms and thus mediate increases in intracellular cAMP. In addition, it was found to be highly homologous (88%) to $G\alpha_s$. Interestingly, $G\alpha_{olf}$ appears to be more widely expressed than first thought and has been detected in human digestive and urogenital epithelial cells (Regnauld et al., 2002) and rat placenta (Itakura et al., 2006). Thus, this $G\alpha$ subunit may be involved in the regulation of more physiological functions than are currently appreciated.

Family	Subunit	Effector	Reference
$G\alpha_s$	$G\alpha_s^*$	Adenylyl cyclases \uparrow	(Northup et al., 1983)
		Src Tyr kinases \uparrow	(Ma et al., 2000)
		GTPase of tubulin \uparrow	(Roychowdhury et al., 1999)
	$G\alpha_{olf}$	Adenylyl cyclases \uparrow	(Jones and Reed, 1989)

Table 1.1 Primary effectors of $G\alpha_s$ subunits. Table compiled from Neves et al., 2002. G protein pathways. *Science*, **296**, 1636-1639; Cabrera-Vera et al., 2003. Insights into G protein structure, function and regulation. *Endocr Rev*, **24**, 765-781; Wettschureck and Offermanns, 2005. Mammalian G proteins and their cell type specific functions. *Physiol Rev*, **85**, 1159-1204; Milligan and Kostenis, 2006. Heterotrimeric G proteins: a short history. *Br J Pharmacol*, **147**, s46-s55 and references shown above. * $G\alpha_s$ has two splice variants ($G\alpha_{s(S)}$ and $G\alpha_{s(L)}$). \uparrow , enhances function.

1.1.4.1.2 $G\alpha_{i/o}$

In the early studies, it was recognised that certain hormones (such as dopamine) could inhibit adenylyl cyclase and thus cause a resultant decrease in cAMP levels (DeCamilli et al., 1979). However, the exact mechanism by which the inhibition of this enzyme came about was not fully understood. Work by Katada and colleagues demonstrated that islet activating protein (IAP) (now commonly known as pertussis toxin (PTX)) dramatically decreases the α_2 -adrenergic receptor-mediated inhibition of glucose-induced insulin release (Katada and Ui, 1981). Furthermore, the same group later showed that IAP is able to ADP ribosylate a 41 kDa membrane protein (Katada and Ui, 1982). Purification of the IAP substrate from the rabbit liver by Bokoch and colleagues identified it as the $G\alpha_i$ subunit (Bokoch et al., 1984). Additionally, electrophoretic separation revealed that a 35 kDa protein and a 10 kDa protein were also present in both the G_i and the G_s preparations used by the authors, at the time suggesting that these G proteins were highly similar to G_t .

The $G\alpha_{i1-13}$ subunits of the $G_{i/o}$ family are widely and highly expressed. Due to their high expression levels, GPCR-dependent activation of these $G\alpha$ subunits has been postulated to be the major facilitator of $G\beta\gamma$ complex-mediated signalling. Functionally, they are frequently studied using PTX in second messenger experiments. Indeed, PTX may ADP ribosylate most $G\alpha_{i/o}$ subunits, with the exception of $G\alpha_z$, on a Cys residue close to their carboxy-terminus (Locht and Antoine, 1995). This ribosylation results in the uncoupling of the G protein α

subunit from the GPCR. It was this sensitivity to PTX that resulted in the naming of $G\alpha_{o/other}$. Huff and colleagues purified two proteins, each of approximately 40 kDa, from the bovine cerebral cortex which were substrates for ADP ribosylation by PTX (Huff et al., 1985). Moreover, Pines and colleagues demonstrated the differential reactivity of antisera raised against $G\alpha_{t1}$ to them (Pines et al., 1985). These data then suggested that, as PTX was able to ADP ribosylate both proteins but that they were immunologically different, they were distinct yet related $G\alpha$ subunits. $G\alpha_{o/other}$ was so named as its function was then unclear.

Several GPCRs such as the M_2 -muscarinic receptor, the D_2 dopamine receptor and the prostaglandin EP_3 receptor are known to directly interact with the $G\alpha_{i/o}$ subunits. Additionally, these $G\alpha$ subunits mediate the activity of a wide variety of intracellular effectors (table 1.2). Most are well established as inhibitors of the activity of adenylyl cyclase and thus cause a reduction in intracellular cAMP (Taussig et al., 1994, Kozasa and Gilman, 1995). Interestingly, the $G\alpha_{o/other}$, the $G\alpha_t$, the $G\alpha_z$ and the $G\alpha_{g/gust}$ $G\alpha$ subunits possess a more restricted expression pattern than $G\alpha_{i1-i3}$ suggesting that they may have highly specialised functions within their respective tissues. Indeed, $G\alpha_t$ is highly expressed in the retina and has been shown to potently activate phosphodiesterase enzymes resulting in the closure of Na^+ channels (Fung et al., 1981).

Family	Subunit	Effector	Reference
$G\alpha_{i/o}$	$G\alpha_{i1-i3}$	Adenylyl cyclases ↓	(Taussig et al., 1994)
		Src Tyr kinases ↑	(Ma et al., 2000)
		Rap1GAP isoform 2 recruitment ↑	(Mochizuki et al., 1999)
		Binds GRIN	(Chen et al., 1999)
		PI3 kinase isoform γ ↑	(Takeda et al., 1999)
	$G\alpha_{o/other}^*$	Adenylyl cyclases ↓	(Taussig et al., 1994)
		Rap1GAP isoform 1 sequestration ↓	(Jordan et al., 1999)
		Binds GRIN	(Chen et al., 1999)
	$G\alpha_{t1-t2}$	cGMP PDE ↑	(Fung et al., 1981)
	$G\alpha_z$	Adenylyl cyclases ↓	(Kozasa and Gilman, 1995)
		Binds GRIN	(Chen et al., 1999)
	$G\alpha_{g/gust}$	Unknown	

Table 1.2 Primary effectors of $G\alpha_{i/o}$ subunits. Table compiled from Neves et al., 2002. G protein pathways. *Science*, **296**, 1636-1639; Cabrera-Vera et al., 2003. Insights into G protein structure, function and regulation. *Endocr Rev*, **24**, 765-781; Wettschureck and Offermanns, 2005. Mammalian G proteins and their cell type specific functions. *Physiol Rev*, **85**, 1159-1204; Milligan and Kostenis, 2006. Heterotrimeric G proteins: a short history. *Br J Pharmacol*, **147**, s46-s55 and references shown above. * $G\alpha_o$ has two splice variants ($G\alpha_{o1/A}$ and $G\alpha_{o2/B}$). GRIN, G protein regulated inducer of neurite outgrowth; cGMP PDE, cyclic guanosine monophosphate phosphodiesterase. ↑, enhances function; ↓, reduces function.

1.1.4.1.3 $G\alpha_{q/11}$

The $G\alpha_{q/11}$ pathway is the classical signalling pathway that stimulates the activity of phospholipase C β (PLC β) to produce the intracellular messengers inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ triggers Ca²⁺ release from intracellular stores and DAG recruits PKC to the membrane and activates it. $G\alpha_q$ was purified by Taylor and colleagues and successfully characterised as a PTX insensitive 42 kDa PLC β activator (Taylor et al., 1990). Evidence suggests that the $G\alpha_{q/11}$ subunits share a differential ability to activate each of the PLC β isoforms. Indeed, the rank order of potency of such activation has been shown to be PLC $\beta_1 \geq$ PLC $\beta_3 >$ PLC β_2 (Smrcka and Sternweis, 1993). PLC β_4 is also activated by $G\alpha_{q/11}$ but the basal activity of this enzyme is significantly decreased by ribonucleotides thus making the determination of the extent of the activation difficult (Lee et al., 1994). In addition to the modulation of the activity of PLC β , $G\alpha_{q/11}$ may also directly affect the activity of other intracellular effectors (table 1.3). Recently, Lutz

and colleagues identified p63 RhoGEF as a novel direct downstream effector of these $G\alpha$ subunits (Lutz et al., 2005). The authors demonstrated that the effect of this protein on Rho-induced transcriptional activity is mediated by protein-protein interactions between $G\alpha_{q/11}$ and the p63 RhoGEF PH domain. This study therefore provided a direct link between the $G_{q/11}$ -coupled GPCRs and the regulation of the activity of the Rho monomeric G proteins.

Family	Subunit	Effector	Reference
$G\alpha_{q/11}$	$G\alpha_q$	PLC β s \uparrow	(Lee et al., 1992a)
		BTK \uparrow	(Ma and Huang, 1998)
		p63 RhoGEF \uparrow	(Lutz et al., 2005)
	$G\alpha_{11}$	PLC β s \uparrow	(Lee et al., 1992a)
		p63 RhoGEF \uparrow	(Lutz et al., 2005)
	$G\alpha_{14}$	PLC β s \uparrow	(Lee et al., 1992a)
	$G\alpha_{15/16}^*$	PLC β s \uparrow	(Lee et al., 1992a)

Table 1.3 Primary effectors of $G\alpha_{q/11}$ subunits. Table compiled from Neves et al., 2002. G protein pathways. *Science*, **296**, 1636-1639; Cabrera-Vera et al., 2003. Insights into G protein structure, function and regulation. *Endocr Rev*, **24**, 765-781; Wettschureck and Offermanns, 2005. Mammalian G proteins and their cell type specific functions. *Physiol Rev*, **85**, 1159-1204; Milligan and Kostenis, 2006. Heterotrimeric G proteins: a short history. *Br J Pharmacol*, **147**, s46-s55 and references shown above. * $G\alpha_{15}$ and $G\alpha_{16}$ are mouse and human orthologues respectively (Wilkie et al., 1991). BTK, Brutons Tyr kinase. \uparrow , enhances function.

Both the $G\alpha_q$ and the $G\alpha_{11}$ subunits are almost ubiquitously expressed while the other $G\alpha$ subunits of this family show a more restricted expression pattern. Their biological importance is well established as evidenced by the fact that the deletion of both the $G\alpha_q$ and the $G\alpha_{11}$ genes results in mouse embryonic lethality (embryonic day 10.5) arising from a defect in cardiomyocyte proliferation (Offermanns et al., 1998). Interestingly, given that $G\alpha_{15}$ is primarily expressed in haematopoietic cells, $G\alpha_{15}$ knockout mice show normal haematopoietic and inflammatory responses (Davignon et al., 2000). Thus, the physiological significance of these $G\alpha$ subunits is not currently clear. $G\alpha_{14}$ is primarily expressed in the kidney, the lung and the spleen. As with $G\alpha_{15/16}$, the physiological relevance of this $G\alpha$ subunit is currently undefined. A recent study demonstrated that $G\alpha_{14}$ activation can lead to the activation of signal transducer and activator of transcription 3 (STAT3) (Lo and Wong, 2004). Furthermore, STAT3 has been characterised as a potent oncogenic

molecule that plays a role in the formation of lung adenocarcinomas (Qu et al., 2009). These data, when viewed collectively, may suggest a role for $G\alpha_{14}$ in the pathological context.

Direct pharmacological inhibition of the $G\alpha_{q/11}$ G protein subunits has only recently become possible. Takasaki and colleagues developed YM-254890, a specific $G\alpha_{q/11}$ inhibitor (Takasaki et al., 2004). The authors showed that this compound potently and completely inhibits $G\alpha_{q/11}$ -induced gene transcription. Furthermore, the mechanism by which this inhibition comes about was characterised and shown to involve blockade of GDP/GTP exchange. GDP exchange for GTP is, as reviewed, essential for $G\alpha$ subunit activation and consequent downstream signalling. This inhibitor therefore represents a powerful tool for the study of $G\alpha_{q/11}$ -mediated signalling and, as such, has been used in several of the studies detailed within this thesis.

1.1.4.1.4 $G\alpha_{12/13}$

Of the four G protein families, the $G_{12/13}$ family is now perhaps the most intensively studied. The two members, G_{12} and G_{13} , were discovered through sequence homology (< 45%) with known $G\alpha$ subunits and are expressed ubiquitously (Strathmann and Simon, 1991, Milligan et al., 1992). They have received considerable attention as regulators of both cellular morphology and gene transcription. Indeed, constitutively active forms of both α subunits stimulate actin stress fibre formation and focal adhesion assembly in quiescent Swiss 3T3 cells (Buhl et al., 1995). Similarly, in $G\alpha_{q/11}$ knockout MEF cells, agonist stimulation of the serotonin 5-HT_{2C} receptor leads to the rapid formation of stress fibres in a Rho-dependent manner (Gohla et al., 1999). In $G\alpha_{13}$ knockout MEF cells, stimulation of the same receptor has no cytoskeletal effect. At the level of gene transcription, Mao and colleagues demonstrated that constitutively active $G\alpha_{12}$ ($G\alpha_{12}Q231L$), $G\alpha_{13}$ ($G\alpha_{13}Q226L$), Rho (RhoG14V) and cdc42 (cdc42G12V) induce serum response element- (SRE) promoted luciferase activity (Mao et al., 1998a). Furthermore, specific inhibition of Rho with *C. botulinum* C3 transferase (C3) completely inhibits the luciferase signal induced by the $G\alpha_{12}$, the $G\alpha_{13}$ and the Rho mutants. More

recently, Orth and colleagues showed that *P. multocida* toxin (PMT) induces SRE-driven transcription in HEK293 cells (Orth et al., 2005). Inhibition with either dominant negative $G\alpha_{13}$ ($G\alpha_{13}G225A$) or YM-254890 significantly reduces the PMT-mediated luciferase signal. The authors went on to show that PMT stimulates Rho activation in a $G\alpha_{q/11}$ knockout MEF cell line and that this activation is not inhibited by YM-254890.

The early studies described previously implied that a link existed between $G\alpha_{12/13}$ and Rho. Subsequently this was confirmed and the activation of Rho was shown to involve the interaction of $G\alpha_{12}$ and $G\alpha_{13}$ with RhoGEFs. Hart and colleagues demonstrated that $G\alpha_{13}$ and, to a lesser extent, $G\alpha_{12}$ bind to p115 RhoGEF (Hart et al., 1998). Furthermore, $G\alpha_{13}$, but not $G\alpha_{12}$, stimulates its capacity to catalyse guanine nucleotide exchange on and thus activation of Rho. Similarly, Fukuhara and colleagues showed that the Lsc homology domain of PDZ RhoGEF forms stable complexes with $G\alpha_{12}$ and $G\alpha_{13}$ and suggested that this RhoGEF mediates the activation of Rho by these G protein α subunits (Fukuhara et al., 1999). More recently, Suzuki and colleagues characterised the interaction of leukaemia-associated RhoGEF (LARG) with $G\alpha_{12}$ and $G\alpha_{13}$ (Suzuki et al., 2003). Analogous to earlier results obtained with p115 RhoGEF (Kozasa et al., 1998), the RGS domain of LARG was shown to possess GAP activity for both $G\alpha_{12}$ and $G\alpha_{13}$. Furthermore, LARG activity was demonstrated to be stimulated by constitutively active $G\alpha_{13}$ ($G\alpha_{13}Q226L$) and, when LARG is Tyr phosphorylated by Tec kinase, constitutively active $G\alpha_{12}$ ($G\alpha_{12}Q229L$). These data therefore provide strong evidence to indicate that the $G_{12/13}$ G proteins positively regulate Rho. It is important to note, however, that such regulation by GPCRs is not limited to $G_{12/13}$ -coupled receptors. Vogt and colleagues showed that $G\alpha_{q/11}$ also activates Rho in PTX treated $G\alpha_{12/13}$ knockout cells (Vogt et al., 2003). Given that the potency of this activation is approximately twenty-fold lower than that observed in PTX treated $G\alpha_{q/11}$ knockout cells, the authors hypothesised that RhoGEF proteins are less sensitive to regulation by the $G\alpha_{q/11}$ subunits than by the $G\alpha_{12/13}$ subunits. The $G\alpha_{12/13}$ subunits along with their primary effectors are summarised in table 1.4.

Family	Subunit	Effector	Reference
$G\alpha_{12/13}$	$G\alpha_{12}$	PDZ RhoGEF \uparrow	(Fukuhara et al., 1999)
		LARG* \uparrow	(Suzuki et al., 2003)
		RasGAP isoform 1 \uparrow	(Jiang et al., 1998)
		BTK \uparrow	(Jiang et al., 1998)
	$G\alpha_{13}$	PDZ RhoGEF \uparrow	(Fukuhara et al., 1999)
		LARG \uparrow	(Suzuki et al., 2003)
		Radixin \uparrow	(Vaiskunaite et al., 2000)
		p115 RhoGEF \uparrow	(Hart et al., 1998)

Table 1.4 Primary effectors of $G\alpha_{12/13}$ subunits. Table compiled from Neves et al., 2002. G protein pathways. *Science*, **296**, 1636-1639; Cabrera-Vera et al., 2003. Insights into G protein structure, function and regulation. *Endocr Rev*, **24**, 765-781; Wettschureck and Offermanns, 2005. Mammalian G proteins and their cell type specific functions. *Physiol Rev*, **85**, 1159-1204; Milligan and Kostenis, 2006. Heterotrimeric G proteins: a short history. *Br J Pharmacol*, **147**, s46-s55 and references shown above. * $G\alpha_{12}$ only stimulates the RhoGEF activity of LARG when LARG is Tyr phosphorylated by Tec kinase. BTK, Brutons Tyr kinase. \uparrow , enhances function.

Interestingly, there is an extremely strong trend for coincident coupling of a GPCR to members of the $G_{12/13}$ and the $G_{q/11}$ G protein families. Additionally, synchronised signalling by both $G_{q/11}$ and $G_{12/13}$ has previously received attention at the level of gene transcription, cellular transformation and the activation of the mitogen activated protein kinase (MAPK) cascades (Fukuhara et al., 2000, Gratacap et al., 2001, Marinissen et al., 2003, Orth et al., 2005). Such a strong trend in GPCR coupling specificity has been proposed to suggest that certain GPCR structural elements that underpin G protein activation may be conserved between $G_{q/11}$ - and $G_{12/13}$ -coupled GPCRs (Riobo and Manning, 2005). This hypothesis, however, is difficult to reconcile with the fact that not all $G_{q/11}$ -coupled receptors interact with $G_{12/13}$ and, reciprocally, not all $G_{12/13}$ -coupled receptors interact with $G_{q/11}$.

The $G\alpha_{12/13}$ subunits have frequently been studied in relation to cellular proliferation, migration and transformation (Kelly et al., 2007). It is unsurprising then that, pathologically, they have implied roles in tumour cell invasion and metastasis. Kelly and colleagues demonstrated that $G\alpha_{12}$ is more highly expressed in invasive prostate adenocarcinomas than in benign prostate tissue and that expression of constitutively active $G\alpha_{12/13}$ mutants ($G\alpha_{12}Q231L$ and $G\alpha_{13}Q226L$ respectively) in prostate cancer

cell lines increases cell invasion in a Rho-dependent manner (Kelly et al., 2006b). Additionally, the authors documented similar findings with regards to breast cancers (Kelly et al., 2006a). Constitutively active $G\alpha_{12/13}$ ($G\alpha_{12}Q231L$ and $G\alpha_{13}Q226L$ respectively) increases breast cancer invasion *in vitro*, inhibition of $G\alpha_{12/13}$ reduces metastatic dissemination of breast cancer cells *in vivo* and $G\alpha_{12}$ is more highly expressed in invasive adenocarcinomas of the breast. These proposed pathological roles in what are, generally, migratory cellular responses are reflected well in work by Stefan Offermanns (Offermanns et al., 1997). Disruption of *GNA13*, the gene encoding $G\alpha_{13}$, was shown to result in mouse germ line embryonic lethality as a result of a failure to develop a functional vascular system due to impaired angiogenesis. Significantly, *in vitro* these data were shown to correlate with the complete abrogation of thrombin-induced cell migration in $G\alpha_{13}$ knockout cells suggesting that $G\alpha_{13}$ is an absolute requirement for angiogenesis to occur.

1.1.4.2 $G\beta\gamma$

The $G\beta$ subunit possesses an amino-terminal α helix followed by a β propeller structure composed of seven β sheets (Lambright et al., 1996, Dupre et al., 2008). Each β sheet is made up of four antiparallel β strands and the structure is linked by WD repeats. The $G\gamma$ subunit is made up of two α helices. The amino-terminal α helix forms a coiled-coil with the amino-terminal α helix of the $G\beta$ subunit and the carboxy-terminal α helix interacts with the β propeller region. The interaction between the $G\alpha$ subunit and the $G\beta\gamma$ complex involves the interaction of residues within the switch regions of the $G\alpha$ subunit (which are flexible in the $G\alpha$ -GDP inactive conformation) with residues at the top of the β propeller domain. Additionally, residues in the amino-terminal α helix of the $G\alpha$ subunit interact with residues on the side of the β propeller.

Historically, the $G\beta\gamma$ complex was regarded as little more than a passive membrane anchor of the $G\alpha$ subunit. Consistent with this role, isoprenylation of the $G\gamma$ subunit at a Cys residue in the carboxy-terminal tail is thought to be essential for membrane localisation (Clapham and Neer, 1997). However, evidence now suggests that the $G\beta\gamma$ complex, when freed from the $G\alpha$ -GTP subunit, can regulate the activity of

various intracellular effectors (table 1.5). The first experimental verification of this came to light when Logothetis and colleagues demonstrated that the G $\beta\gamma$ complex activates a K⁺ channel in cardiac atrial cells (Logothetis et al., 1987). Since then, a plethora of different effectors regulated by the G $\beta\gamma$ complex has been uncovered. Upon GPCR activation, conformational changes in the switch regions of the G α subunits reduce their affinity for the G β subunits and promote the dissociation of G α -GTP and the G $\beta\gamma$ complex (Lambright et al., 1994, Lambright et al., 1996).

Family	Effector	Reference
G $\beta\gamma$	PLC β s \uparrow	(Camps et al., 1992)
	BTK \uparrow	(Langhans-Rajasekaran et al., 1995)
	Adenylyl cyclase isoforms 1, 5, 6 and 8 \downarrow	(Tang and Gilman, 1991) (Bayewitch et al., 1998) (Steiner et al., 2006)
	Adenylyl cyclase isoforms 2, 4 and 7 \uparrow	(Tang and Gilman, 1991) (Gao and Gilman, 1991) (Yoshimura et al., 1996)
	K ⁺ channels \uparrow	(Logothetis et al., 1987)
	Ca ²⁺ channels \downarrow	(Ikeda, 1996)
	PI3 kinases \uparrow	(Stephens et al., 1994) (Tang and Downes, 1997)
	Protein kinase D \uparrow	(Jamora et al., 1999)
	Ca ²⁺ -calmodulin-mediated activation of calmodulin kinase isoform 2 \downarrow	(Liu et al., 1997)
	Tsk kinase \uparrow	(Langhans-Rajasekaran et al., 1995)
	GTPase of dynamin 1 \uparrow	(Lin and Gilman, 1996)
	Src Tyr kinases \uparrow	(Shajahan et al., 2004) (Gentili et al., 2006)

Table 1.5 Primary effectors of the G $\beta\gamma$ complex. Table compiled from Cabrera-Vera et al., 2003. Insights into G protein structure, function and regulation. *Endocr Rev*, **24**, 765-781; Wettschureck and Offermanns, 2005. Mammalian G proteins and their cell type specific functions. *Physiol Rev*, **85**, 1159-1204; Milligan and Kostenis, 2006. Heterotrimeric G proteins: a short history. *Br J Pharmacol*, **147**, s46-s55 and references shown above. BTK, Brutons Tyr kinase. \uparrow , enhances function; \downarrow , reduces function.

1.1.4.3 Monomeric G Proteins

The monomeric G protein family may be subdivided, on the basis of structural similarity, into five subfamilies (Ras, Rho, Rab, ARF and Ran) (Matozaki et al., 2000, Bhattacharya et al., 2004, Bos et al., 2007). In general, the Ras subfamily regulates cell signalling events that culminate in gene transcriptional responses, the Rho subfamily function as actin cytoskeleton regulators and can also mediate gene transcription, the Rab and the ARF subfamilies control vesicular trafficking between different cellular compartments and the Ran subfamily regulates nucleocytoplasmic transport. As with their heterotrimeric counterparts, the monomeric G proteins exist in a GDP-bound inactive state which is converted into an active GTP-bound state by the association of a guanine nucleotide exchange factor (GEF) (the activity of which is regulated by an upstream signal) (Takai et al., 2001). GTP binding induces a conformational change in the effector binding domain of the protein and the interaction with target molecules ensues. Switching off of the system is mediated by the intrinsic GTPase activity of the monomeric G protein and the subsequent hydrolysis of GTP to GDP. Interaction with a GAP accelerates this process.

The major effectors of the Ras subfamily are Raf kinases, phosphatidylinositol-3-kinase (PI3 kinase) isoforms and RalGEFs (Shields et al., 2000, Eisenberg and Henis, 2008). In regulating these and other signalling molecules, Ras monomeric G proteins control processes involved in cell growth, proliferation, differentiation and, enigmatically, apoptosis (Cox and Der, 2003). Thus, Ras regulation of cell cycle progression is complex and is thought to involve the interplay of multiple signalling cascades. Indeed, constitutively active Ras induces a proliferative phenotype in human thyroid epithelial cells *in vitro* (Gire et al., 2000). Conversely, Ras has been demonstrated to effectively promote apoptosis through the Raf pathway (Kauffmann-Zeh et al., 1997). These data therefore suggest that the combined outcome of Ras-induced signalling is likely to determine final cellular fate.

Like Ras, the Ras homologue (Rho) proteins serve as key regulators of extracellular stimulus-mediated signalling and regulate cytoskeletal reorganisation, cell cycle progression and gene expression. The best characterised members of this subfamily

are Rho, Rac and cdc42 (Wennerberg et al., 2005). With reference to the regulation of the actin cytoskeleton, Rho mediates the formation of stress fibres and focal adhesions (Ridley and Hall, 1992) while Rac induces the formation of lamellipodia (Ridley et al., 1992). Cdc42 regulates the formation of filopodia (Nobes and Hall, 1995). For some time now, it has been appreciated that Rho may be inhibited by Rac/cdc42 and, conversely, Rac/cdc42 may be inhibited by Rho. The molecular mechanisms underlying this mutual antagonism are poorly understood. Sander and colleagues demonstrated that sustained Rac/cdc42 activity results in the inhibition of the activity of Rho and correlates with decreased levels of Rho-GTP (Sander et al., 1999). The authors speculated that the increased activity of a RhoGAP or the decreased activity of a RhoGEF was central to their observations as the inhibition of Rho activity appeared to occur either upstream of or directly at the monomeric G protein level. Their conclusions are supported by an earlier study by Leeuwen and colleagues which showed that the Rac-induced inhibition of Rho signalling is completely overcome by the expression of constitutively active Rho (Leeuwen et al., 1997). These data therefore imply that GTP loading on Rho is the target of the Rac-/cdc42-mediated negative regulation. More recently, and in agreement with this suggestion, Nimnual and colleagues provided evidence to suggest that the Rac-induced production of reactive oxygen species inhibits the low molecular weight protein Tyr phosphatase and thus increases the Tyr phosphorylation of p190 RhoGAP (Nimnual et al., 2003). Furthermore, it has been reported that p21 activated kinase 1 (Pak1) binds, in a Rac-dependent manner, to p115 RhoGEF and thus disrupts thrombin-induced Rho activation by an as yet unknown mechanism (Rosenfeldt et al., 2006). Finally, Barac and colleagues demonstrated that PAK4, a cdc42 effector, directly interacts with and phosphorylates PDZ RhoGEF (Barac et al., 2004). Such phosphorylation results in the abolishment of the ability of this RhoGEF to mediate $G\alpha_{12/13}$ -induced GTP loading on Rho.

As highlighted in the previous paragraph, it is now also generally accepted that Rac/cdc42 may be inhibited by Rho. One of the earliest pieces of evidence to support this interpretation came from the work of Leeuwen and colleagues in 1997 (Leeuwen et al., 1997). The authors demonstrated that Rac-induced neurite

formation is potentiated by transfecting a dominant negative Rho mutant into an NIE115 neuroblastoma cell line. S19NRho, the dominant negative the authors used, is thought to inhibit endogenous Rho function by forming unproductive complexes with RhoGEFs and thereby halting GDP/GTP exchange (Hart et al., 1998). More recently, Yamaguchi and colleagues showed that transfection of constitutively active $G\alpha_{12}$ or Rho ($G\alpha_{12}Q231L$ or RhoG14V respectively), or incubation with Y-27632 (an inhibitor of Rho kinase (ROCK)), completely abolishes the Rho-induced inhibition of Rac in PC12 cells (Yamaguchi et al., 2001). These results suggest that Rho-mediated inhibition of Rac occurs downstream of Rho but is reliant on Rho-GTP loading. Furthermore, they imply that ROCK may play a central role. The latter part of this suggestion is in contrast to a recent paper in which ROCK was shown to be uninvolved in the Rho-induced Rac inhibition in vascular smooth muscle cells (Takashima et al., 2008). Thus, the Rac- and the Rho-mediated pathways oppose each other by a mechanism which still remains to be fully elucidated. It is recognised, however, that the balance between these monomeric G proteins plays a prominent role in the regulation of cellular morphology.

Rab monomeric G proteins regulate vesicular protein transport during export trafficking, endocytosis, exocytosis and endosome fusion. They constitute the largest group of monomeric G proteins and each member has a distinct intracellular localisation (Zerial and McBride, 2001). Multiple members of this subfamily have been reported to regulate transport of GPCRs from the ER to the Golgi as well as their trafficking between early, late and recycling endosomes and lysosomes (Seachrist and Ferguson, 2003). Indeed, the β_2 -adrenergic receptor has been shown to colocalise with Rab5 in early endosomes following isoproterenol stimulated internalisation (Moore et al., 1995). Furthermore, expression of Rab5Q79L (a constitutively active Rab5 mutant) has been demonstrated to significantly increase the rate of dopamine-induced D_2 receptor endocytosis (Iwata et al., 1999). Rab5S34N (a dominant negative Rab5 mutant) had the opposite effect on both the D_2 dopamine receptor (Iwata et al., 1999) and the β_2 -adrenergic receptor (Seachrist et al., 2000). Expression of a dominant negative Rab4 mutant (Rab4N121I) prevents plasma membrane recycling of the β_2 -adrenergic receptor from endosomes (Seachrist

et al., 2000) and expression of a Rab7 dominant negative mutant (Rab7T22N) prevents the lysosomal targeting of the human CXCR₂ chemokine receptor after prolonged agonist exposure (Fan et al., 2003). These data thus confirm that the Rab family of monomeric G proteins are intricately involved in the regulation of GPCR trafficking.

The ARF subfamily of monomeric G proteins may associate with rhodopsin subfamily GPCRs which possess the NPxxY motif at the intracellular end of TM7 (Mitchell et al., 1998). They are thought to play an essential role in regulating membrane trafficking events involved in endocytosis. Additionally, accumulating evidence indicates that the ARF proteins are important regulators of the actin cytoskeleton and are involved in the regulation of cell adhesion, migration and neurite outgrowth (Myers and Casanova, 2008). One way in which ARFs are thought to influence the actin cytoskeleton is through their effects on the lipid microenvironment. All ARFs are activators of phospholipase D (PLD) which cleaves phosphatidylcholine to generate phosphatidic acid (PA). PA alters the physical properties of membrane bilayers and, as such, facilitates membrane bending at sites of vesicle formation. Additionally, some studies suggest that PA may stimulate stress fibre formation (Ha and Exton, 1993) or play a role in membrane ruffling (O'Luanaigh et al., 2002). Members of the ARF subfamily have been found in all eukaryotic organisms examined to date and show a high degree of sequence conservation (Nie et al., 2003).

Ran proteins are known for their role in nucleocytoplasmic transport of both ribonucleic acid (RNA) and protein molecules. Unlike other monomeric G proteins, their function is dependent on a spatial gradient of the active GTP-bound form (Weis, 2003). There is a high concentration of Ran-GTP in the nucleus which facilitates the directionality of nuclear import and export by direct interaction with members of the importin and exportin protein family of related transport carriers respectively. Additionally, Ran has been implicated in the regulation of mitotic spindle assembly, DNA replication and nuclear envelope assembly (Li et al., 2003).

1.1.5 GPCR-Mediated G Protein-Independent Signalling

Increasing evidence indicates that GPCRs may relay signals independently of heterotrimeric G protein activation. Indeed, it has been shown that they may interact with non-G protein effectors such as the Janus kinases (JAKs) (Ali et al., 2000) and the src family Tyr kinases (Cao et al., 2000). GPCR-mediated regulation of the src family Tyr kinases will, in this section, be briefly discussed.

Src is a non-receptor Tyr kinase involved in the regulation of many physiological responses including cell proliferation, survival, differentiation, adhesion and migration (Sun et al., 2007b). It is a member of the src family of protein Tyr kinases (SFK) along with lyn, hck, lck, blk, fyn, yes and fgr. All members of the SFK share a similar domain arrangement and possess an amino-terminal region followed by a src homology 3 (SH3) domain, a src homology 2 (SH2) domain, a kinase or src homology 1 (SH1) domain and a carboxy-terminal tail (Ingley, 2008). The activity of src is highly regulated by intramolecular interactions involving the SH3, the SH2 and the kinase domains. Inactive src exists in a conformation where the SH3 and the SH2 domains fold back against the kinase domain making intramolecular interactions that lock the kinase in the inactive conformation (Boggon and Eck, 2004). Src activation is initiated by engagement of an activating protein with either the SH2 or the SH3 domain. This results in the disruption of the intramolecular constraints within the molecule. Additionally, dephosphorylation of the autoinhibitory Tyr residue in the carboxy-terminal tail results in kinase activation. These activation events enable autophosphorylation of a Tyr residue within the kinase domain and the consequent induction of full catalytic activity.

It is widely accepted that src can be activated by many of the canonical signalling pathways initiated by GPCRs including those mediated by arrestins. Indeed, the neurokinin NK₁ receptor has been shown to, upon substance P stimulation, form a scaffolding complex comprising the internalised receptor, β -arrestin and src (DeFea et al., 2000). Similarly, the interleukin 8 activated CXCR₁ receptor stimulates the rapid formation of β -arrestin complexes with hck and fgr (Barlic et al., 2000). Formation of β -arrestin-hck complexes leads to hck activation. These data therefore

provide evidence to indicate that GPCR-arrestin complexes may interact with and activate members of the SFK. However, an evolving body of evidence suggests that src may associate with, and be activated directly by, the intracellular domains of certain GPCRs independently of G proteins or arrestin scaffolds. In line with this, based on the observation that src activation by the β_2 -adrenergic receptor is possible in PTX treated $G\alpha_s$ and β -arrestin knockout cells, taken together with the finding that this GPCR activates src in an *in vitro* system in the absence of G proteins, Sun and colleagues proposed that the β_2 -adrenergic receptor-mediated activation of src is initiated by the formation of a direct interaction between the two signalling proteins (Sun et al., 2007a). Fan and colleagues earlier suggested a similar scheme (Fan et al., 2001). Moreover, the authors proposed that association of src with the β_2 -adrenergic receptor involves an interaction of the GPCR with the src SH2 domain (the carboxy-terminal tail of this receptor possesses a Tyr residue (Y350) flanked by a putative SH2 binding domain). It was suggested that, upon agonist-induced phosphorylation of this Tyr residue, the SH2 binding domain becomes functional and recruits SH2 domain containing partners. Similarly, Yun and colleagues showed that the carboxy-terminus of the serotonin 5-HT₆ receptor interacts with fyn and that this interaction is mediated by the fyn SH3 domain (Yun et al., 2007). Activation of this GPCR leads to the fyn-dependent activation of extracellular signal regulated kinase (ERK). These studies therefore provide support for the suggestion that the GPCR-mediated activation of src and the consequent src-dependent signalling may occur independently of both G protein activation and arrestin binding.

Of particular interest, in relation to the data presented within this thesis, is the cytoskeletal reorganising potential of this Tyr kinase. For example, the protein product of the viral oncogene *v-src* has been shown to facilitate the rapid induction of membrane ruffling (Boschek et al., 1981). More recently, a constitutively active src mutant, in cooperation with Tiam1, was demonstrated to markedly induce the formation of lamellipodia (Servitja et al., 2003). The authors showed that Tiam1, a specific RacGEF, is Tyr phosphorylated by src and that this phosphorylation results in the pronounced elevation of Rac-GTP. Src has additionally been shown to directly Tyr phosphorylate and thus activate FRG, a specific cdc42GEF, (Miyamoto

et al., 2003) and to, together with cdc42, Tyr phosphorylate Vav2, another RacGEF (Kawakatsu et al., 2005). Thus, src is known to promote the activation of Rac. Indeed, laminin-induced activation of Rac has been proposed to be initiated by members of the SFK (Zhou et al., 2007) and, very recently, epidermal growth factor (EGF) has been shown to stimulate Rac activation through a src and PI3 kinase-dependent mechanism (Dise et al., 2008). In the latter study, Rac activation was associated with a doubling in the number of cells with lamellipodial extensions.

Interestingly, the src-mediated regulation of Rac does not appear to be exclusive to the regulation of RacGEF activity. Indeed, members of the SFK have also been shown to negatively regulate a RacGAP, β 2-chimaerin, thereby elevating Rac-GTP levels (Kai et al., 2007). Furthermore, the ability of src to regulate the activity of the members of the monomeric G protein family is not restricted to the modulation of Rac/cdc42. Brandt and colleagues showed that PKC-induced src-dependent actin reorganisation correlates with a reduction in the activity of Rho (Brandt et al., 2002). The link between src and a reduction in Rho activity was revealed to be p190 RhoGAP (which is Tyr phosphorylated and thus activated by src downstream of PKC). It is therefore likely that the SFK enhance Rac activity and inhibit Rho activity through both the activation of RacGEFs and RhoGAPs and the inhibition of RacGAPs. This raises the possibility that GPCRs which bind and stimulate src directly may also promote the activity of Rac and inhibit the activity of Rho in a G protein-independent manner. In so doing, these receptors may play a role in processes such as gene transcription and cytoskeletal reorganisation.

1.1.6 Summary

GPCRs share a common structural architecture, are synthesised and trafficked in similar ways, are transited into their active state by a series of agonist-induced conformational changes and may signal through both G protein-dependent and -independent pathways. In the following section, ligand-induced receptor activation and signalling will be reviewed.

1.2 Ligand-Induced Receptor Activation and Signalling

Early receptor theory was consistent with the notion that a single receptor existed in only two conformations – inactive and active. Agonist binding was proposed to shift the receptor from the inactive to the active conformation. Additionally, it was thought that, once activated, a given GPCR could only modulate the activity of a single signalling cascade. As will be discussed, however, increasing evidence suggests that there exist multiple GPCR conformations which may selectively alter the activity of different intracellular signalling pathways. Furthermore, different ligands can stabilise these different receptor conformations. This phenomenon was originally termed stimulus directed trafficking (Kenakin, 1995). In order to provide a comprehensive review, it is first necessary to briefly revisit how agonist binding initiates receptor activation.

1.2.1 Agonist Binding and Receptor Activation

As described in the preceding sections, binding of an agonist to a GPCR induces a conformational change of the receptor. Current thinking proposes that this change in conformation involves the disruption of intramolecular interactions within the GPCR that constrain the receptor in the inactive state (Gether, 2000, Lu et al., 2002, Kobilka and Deupi, 2007). This disruption may be facilitated by two separate mechanisms. Firstly, agonist binding is simply thought to disrupt the intramolecular restraint network. In support of this theory, experimental evidence has shown that the mutation of specific residues within a GPCR may result in constitutive receptor activity. For example, Lu and colleagues showed that the mutation of Leu¹¹⁶ (L116A) in the M₁-muscarinic receptor results in constitutive receptor activation (Lu and Hulme, 1999). Furthermore, in a subsequent study, the authors suggested that Leu¹¹⁶ interacts with Phe³⁷⁴ and in doing so stabilises the GPCR inactive state (Lu and Hulme, 2000). Thus, disruption of this interaction induces the transition of the receptor to an active conformation. Secondly, agonists are thought to facilitate the creation of new intermolecular and intramolecular interactions. These new interactions stabilise the receptor in an active conformation. Indeed, no constitutive activity was observed upon the mutation of Cys²⁷⁹ (C279A or C279Y) or Asn³¹⁵ (N315A) in the GnRH receptor (the mutation of equivalent residues has previously

been shown to cause the constitutive activation of other GPCRs such as the β_2 -adrenergic receptor (Shi et al., 2002) and the H_1 histamine receptor (Jongejan et al., 2005)) suggesting that disruption of these interactions is not sufficient to obtain a fully active receptor state (Lu et al., 2007). Both mechanisms described above probably occur to some degree at a given receptor but it is thought that one may serve as the dominant activation facilitator.

Interaction between an agonist and a receptor has been suggested to involve an initial interaction between the receptor and one structural group of the agonist (Kobilka, 2004, Swaminath et al., 2004, Kobilka and Deupi, 2007). Binding of the remaining structural groups is proposed to occur in a sequential manner. Specifically, binding of an agonist structural group (A) to a receptor facilitates the formation of a set of energetically favourable interactions between the agonist and the GPCR and thus increases the energetic probability of an interaction between the receptor and a second agonist structural group (B). This interaction in turn increases the energetic probability of an interaction between the GPCR and a third agonist structural group (C). Importantly, each sequential interaction between the receptor and A, B and C results in the stabilisation of a distinct intermediate conformational state. Indeed, monitoring of a fluorescently labelled β_2 -adrenergic receptor revealed the formation of distinct consecutive agonist-induced receptor active conformations over a period of approximately 500 seconds (Swaminath et al., 2004).

GPCRs are therefore not simply constrained to two states (inactive and active) but may, in response to agonist binding, adopt a series of distinct receptor conformations. Thus, the possibility that these agonist-induced conformations have signalling relevance arises. In a recent review, Brian Kobilka elegantly summarises work from his laboratory on the β_2 -adrenergic receptor which confirms this (Kobilka, 2007). These studies, which shall later be discussed, suggest that agonists of the same receptor can stabilise different receptor conformations and thus modulate different intracellular signalling pathways. For the remainder of this review, I will refer to this concept as ligand-induced selective signalling (LiSS) (figure 1.7).

1.2.2 Ligand-Induced Selective Signalling

LiSS is not a new concept and has been extensively reviewed by Terry Kenakin (Kenakin, 1995, Kenakin, 2001, Kenakin, 2003). Over the years, it has variously been referred to as ligand directed trafficking of receptor signalling, stimulus directed trafficking, functional selectivity, biased agonism and agonist-induced signal trafficking (Kenakin, 1995, Galandrin et al., 2007). While the former three terms used to describe this phenomenon are essentially accurate, biased agonism and agonist-induced signal trafficking may be interpreted as being unfortunate misnomers (Millar et al., 2004). Indeed, these terms neglect historical pharmacological nomenclature given that experimental evidence suggests that both agonists and ligands originally classified as antagonists can signal through GPCRs (Yano et al., 1992, Maudsley et al., 2004).

One of the early demonstrations of GPCR conformation-induced selective signalling states came from mutational studies of the α_{1B} -adrenergic receptor (Perez et al., 1996). Mutation of Cys¹²⁸ in TM3 (C128F) constitutively activated the receptor. Furthermore, this constitutive activation was shown to be selective in that it only resulted in the activation of a PLC-mediated pathway and not one mediated by phospholipase A₂ (PLA₂). Previously, the same group had shown that the PLC- and the PLA₂-induced pathways are activated by coupling of the receptor to different G proteins (G_{q/11} and G_{i/o} respectively) (Perez et al., 1993). Similarly, mutation of Cys¹¹⁶ in TM3 of the β_2 -adrenergic receptor (C116F) results in constitutive activation of NHE1, a Na⁺-H⁺ exchanger, without activating a G_s-mediated pathway (Zuscik et al., 1998). Both studies therefore indicate that mutation-induced receptor conformational changes can alter the output of distinct intracellular signalling pathways.

In 2001, Ghanouni and colleagues studied fluorescence lifetime analysis of a fluorophore covalently attached to Cys²⁶⁵ in ICL3, at the intracellular end of TM6, of the β_2 -adrenergic receptor (Ghanouni et al., 2001). The authors demonstrated that a full agonist, isoproterenol, and two partial agonists, salbutamol and dobutamine, provoke different fluorescence lifetime distributions suggesting that they induce

different receptor conformational states. In support of this conclusion, earlier observations by Gether and colleagues revealed that isoproterenol and propranolol could stimulate distinct changes in receptor conformation (Gether et al., 1995). Using a β_2 -adrenergic receptor covalently labelled with a fluorescent probe which was sensitive to the polarity of the surrounding environment, the authors showed that isoproterenol stimulation induces opposing effects on baseline fluorescence.

Taken together, the studies already discussed provide some good examples of the ability of ligands to provoke different conformations of the same GPCR and the ability of mutation-induced changes in receptor conformation to propagate signals through selective downstream signalling pathways. Theoretically, these results therefore suggest that different ligands can selectively modulate the activity of different intracellular signalling pathways by stimulating specific changes in the conformation of a given receptor. Swaminath and colleagues provided convincing evidence to support this proposal (Swaminath et al., 2004). Specifically, distinct agonist-induced receptor conformations were shown to correlate with the activation of discrete signalling cascades. Dopamine, which lacks a hydroxyl group that is present in both norepinephrine and epinephrine, induced only a fast conformational change and stimulated G_s activation but not receptor internalisation. In contrast, both norepinephrine and epinephrine induced a fast and a slow conformational change and stimulated G_s activation and receptor internalisation. Furthermore, a group of ligands (1-2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI) and lysergic acid diethylamide (LSD)) with a similar ability to activate $G_{q/11}$ by binding to the serotonin 5-HT_{2C} receptor display marked differences in their ability to activate G_{i3} (Cussac et al., 2002).

Although GPCRs are renowned for their ability to signal through G proteins, evidence presented earlier in this review indicates that they may also relay signals completely independently of G protein activation. It is important to note, therefore, that the signalling specificity induced by different GPCR conformations is not thought to be confined to the alteration of the activity of the G protein-mediated pathways. Indeed, experimental evidence has shown that while ICI 11851 (an

inverse agonist at the β_2 -adrenergic receptor) decreases both basal cAMP accumulation and cAMP response element- (CRE) mediated gene transcription, stimulation with propranolol (also an inverse agonist) reduces basal cAMP accumulation but stimulates CRE-mediated transcriptional activity (Baker et al., 2003). This observation could not be explained by the conventional G_s -mediated pathway and was found to be insensitive to PTX. Similarly, inhibitors of the $G_{q/11}$ - (Gö6976 or Gö6986) and the $G_{12/13}$ - (Y-27632) mediated signalling pathways were without effect. The authors suggested that propranolol could simultaneously act as an inverse agonist through a G_s -coupled signalling pathway while acting as an agonist through a G protein-independent mechanism. In contrast, ICI 11851 was thought to act as an inverse agonist at both signalling cascades. This study therefore supports the proposal that LiSS is not restricted to the modulation of only the activity of the GPCR-G protein-dependent pathways.

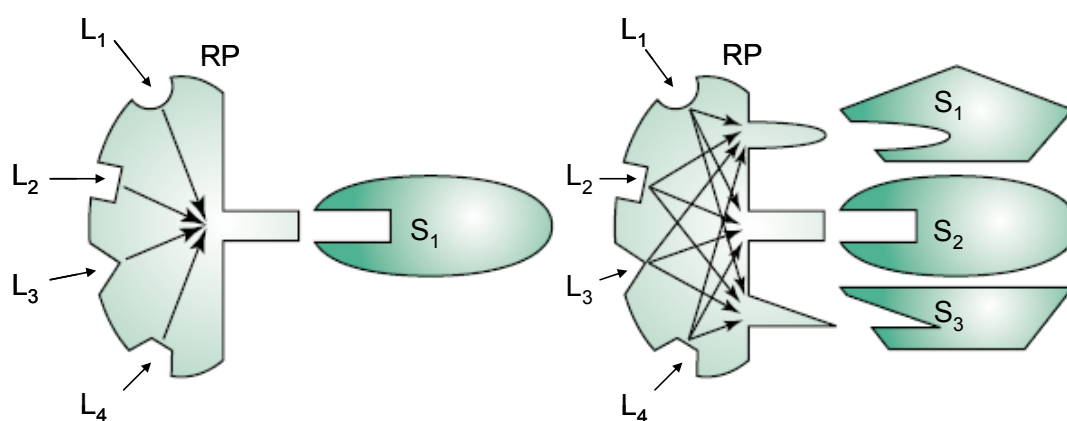


Figure 1.7 The concept of LiSS. Early receptor theory suggested that a single receptor existed in only two states – inactive and active (left panel). Structurally diverse ligands (L_1 , L_2 , L_3 and L_4) bind to the same receptor protein (RP) and modulate the activity of a single downstream signalling pathway (S_1). Increasing evidence, however, suggests that different receptor conformations can selectively alter the output of different intracellular signalling pathways (S_1 , S_2 and S_3) (right panel). Furthermore, structurally diverse ligands can induce distinct changes in receptor conformation. Thus, ligand structure dictates the activity of discrete signalling cascades. Figure adapted from Kenakin, 2003. Ligand selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci*, **24**, 346-354.

The work discussed previously highlights an important limitation of the classical pharmacological classification of ligands. In the study by Baker and colleagues, propranolol was observed to simultaneously act as an inverse agonist through a G_s -coupled signalling pathway while acting as an agonist of the ERK cascade through a G protein-independent mechanism (Baker et al., 2003). Thus, classifying ligands on the basis of their ability to modulate a single effector does not provide a complete and accurate description of their signalling potential. Historically, one measure used to characterise ligands was efficacy. Stephenson first used the term efficacy to denote the property of a ligand that caused it to activate a receptor and produce a pharmacological response (Stephenson, 1956). It is considered to be a proportionality factor designed to quantify the power of agonists (Kenakin, 2002). However, the observation that GPCRs can exhibit various behaviours, including the ability to differentially alter the activity of multiple G proteins and non-G protein effectors, dictates that different ligands may possess dual, no or opposite efficacy at different signalling pathways (figure 1.8). In the example mentioned previously, propranolol has negative efficacy at the β_2 -adrenergic receptor- G_s -mediated signalling pathway as it decreases basal cAMP accumulation. In contrast, it has a positive measure of efficacy at the β_2 -adrenergic receptor- G_s -independent pathway as it stimulates a CRE-mediated transcriptional output. Obviously, in practice, the large number of signalling outputs makes it difficult to represent the efficacy of a compound according to the ensemble of potential signalling pathways it modulates. Thus, most ligand descriptions may be partially inaccurate.

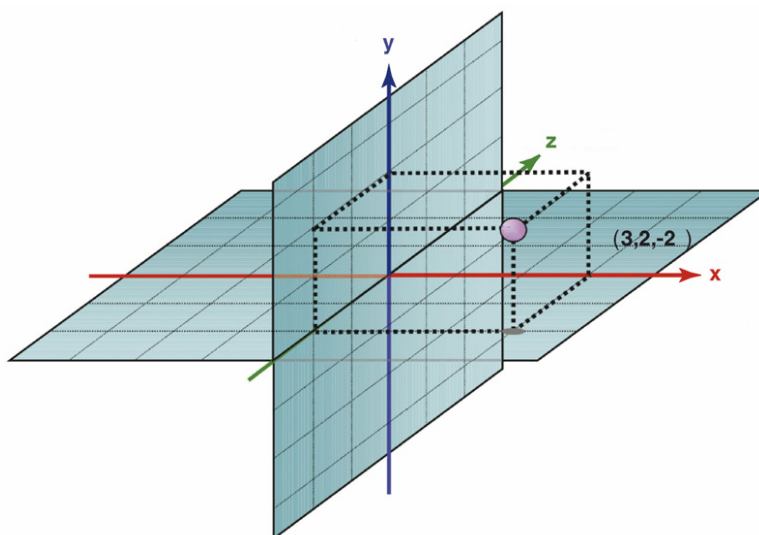


Figure 1.8 Ligands may possess dual, no or opposite efficacy at different signalling pathways. Each axis (x, y and z) represents a specific signalling pathway, the activity of which may be modulated by a GPCR. The compound illustrated possesses a positive efficacy for x and thus activates this pathway. It also possesses a positive efficacy for y and consequently stimulates the relevant downstream effectors. Finally, it has a negative efficacy for z and therefore decreases basal activation of this signalling cascade. Figure adapted from Galandrin et al., 2007. The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci*, **28**, 423-430.

The underlying basis of LiSS is the ability of ligands to promote ligand selective GPCR conformations which have distinct abilities to engage different downstream signalling pathways. However, the specific molecular processes involved still remain elusive. Yao and colleagues postulated that, upon ligand binding, combinations of receptor molecular switches are turned on (Yao et al., 2006). Furthermore, structurally diverse ligands may activate different combinations of these switches and each switch has the ability to alter the conformation of a specific receptor domain. Such switch activation, therefore, results in the ligand specific rearrangement of the conformation of the GPCR. Thus, the resulting receptor conformation differentially modulates distinct downstream signalling pathways. Future research should address precisely which switches are activated in response to different ligands and how this correlates with ligand structure. Additionally, it is essential that the relationship between the activation of specific groups of switches and the modulation of specific downstream signalling pathways is established.

While neither of these goals will be straightforward to achieve, particularly as there will undoubtedly be some variation between individual receptors, the possible pharmacological and therapeutic gains are unquestionably worth the effort.

1.2.3 Summary

LiSS raises the possibility that novel therapeutic agents that modify only portions of the behaviour of a given GPCR may be designed (Kenakin, 2003). Such agents could reduce the spectrum of undesirable pathological effects caused by the activation of a specific receptor while concomitantly potentiating a desirable physiological output. To attain such signalling specificity, LiSS still requires thorough investigation as detailed in the previous section. Additionally, it is necessary that the data gleaned from *in vitro* systems is rigorously assessed in terms of the physiological and the therapeutic relevance and that this assessment takes place in the context of the tissue or organ that is to be pharmacologically targeted.

1.3 GnRH and GnRH Receptor-Mediated Signalling

GnRH is the central initiator of the reproductive hormonal cascade (Millar, 2005) (figure 1.9). It belongs to a group of peptides originally discovered and successfully isolated as factors of hypothalamic origin which control secretions of the anterior pituitary gland (Schneider et al., 2006). It is processed in the hypothalamus by proteolytic cleavage of a precursor polypeptide and packaged into storage granules which are transported down axons to the external zone of the median eminence (Millar et al., 2004). Here, release into the hypophyseal portal circulation occurs in a synchronised pulsatile manner. Such release results in the stimulation of the biosynthesis and the secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Sisk and Foster, 2004). LH and FSH, in turn, regulate gonadal steroidogenesis and gametogenesis in both sexes.

Most vertebrates studied possess at least two forms of GnRH (Morgan and Millar, 2004, Pawson and McNeilly, 2005). The hypothalamic form, GnRH I, is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) and differs from GnRH II by three residues in positions five, seven and eight (Lu et al., 2005, Lu et

al., 2007, Coetsee et al., 2008). GnRH II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂) was originally identified in chicken hypothalami (Miyamoto et al., 1984) and is conserved from teleost fish to humans suggesting that it has critical and specific functions. Interestingly, one of the established biological roles of GnRH II is the inhibition of the M current (a K⁺ current found in many neuronal cell types) in bullfrog sympathetic neurons (Jones, 1987). Historically, the presence of GnRH II in most vertebrates suggested the probable existence of cognate type II GnRH receptors. However, although characterised in some non-human primates such as the marmoset (Millar et al., 2001) and the rhesus monkey (Neill et al., 2001), the human type II GnRH receptor gene is disrupted by a frame shift and premature stop codon (Morgan et al., 2003). Nevertheless, both GnRH I and GnRH II can bind to the human type I GnRH receptor with high affinity and thus this receptor can mediate the actions of both ligands in man.

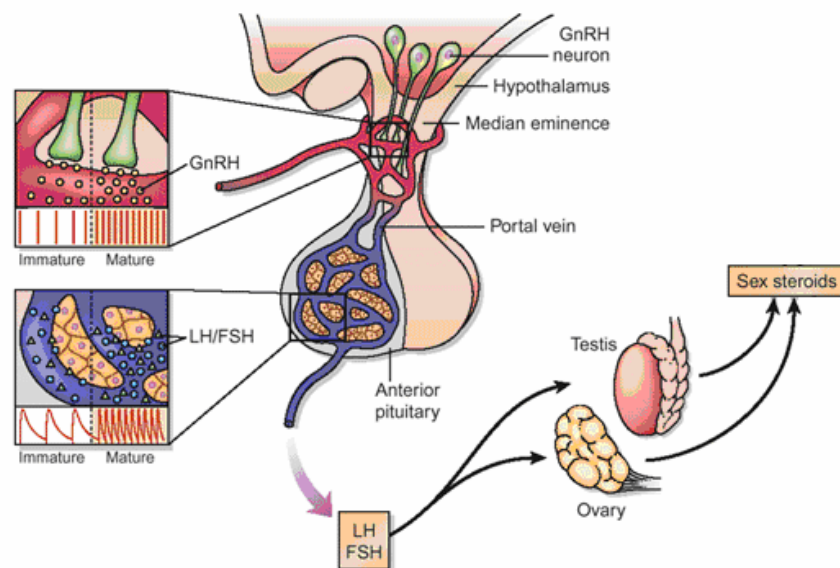


Figure 1.9 The hypothalamic-pituitary-gonadal axis. GnRH is processed in the hypothalamus and packaged into storage granules which are transported down axons to the external zone of the median eminence. Here, release into the hypophyseal portal circulation occurs. This release results in the stimulation of the biosynthesis and the secretion of LH and FSH. These hormones, in turn, regulate gonadal steroidogenesis and gametogenesis. Figure adapted from Sisk and Foster, 2004. The neural basis of puberty and adolescence. *Nat Neurosci*, 7, 1040-1047.

1.3.1 Physiological and Therapeutic Roles of GnRH

GnRH I functions as a physiological regulator of the gonadotropins while GnRH II may be involved in neuromodulation (Millar, 2005). Akin to this function, it has been suggested that GnRH II plays a role in the regulation of sexual behaviour (Millar, 2003). Additionally, both peptides have been implicated in the modulation of a wide variety of other functions in extrapituitary tissues (table 1.6). Clinically, synthetic GnRH analogues are used to influence ovulation in assisted reproduction. Furthermore, chronic stimulation with high doses of GnRH agonists is extensively used in the treatment of hormone-dependent diseases such as endometriosis, uterine fibroids, precocious puberty and sex steroid-dependent cancers. Such stimulation desensitises the gonadotrope causing a resultant decrease in LH and FSH and a decline in ovarian and testicular function. GnRH antagonists also inhibit the reproductive system but the doses required are higher than those for GnRH agonists (Millar et al., 2000). Thus, these compounds present challenges for clinical administration. They do, however, overcome the undesirable agonist-mediated initial stimulation of the reproductive system which lasts several weeks before the onset of desensitisation.

Of interest, in the pathological context, is the frequently reiterated paradigm of the ability of GnRH analogues to directly inhibit the proliferation of reproductive cancer cells. These effects appear to be dependent on the degree of GnRH receptor expression and the intracellular signalling protein milieu (Morgan et al., 2008, White et al., 2008b). Quantification of functional receptor protein has, surprisingly, been determined in relatively few cell types and several groups have demonstrated only receptor expression at the messenger RNA (mRNA) level (Kottler et al., 1997, Bahk et al., 1998, Yin et al., 1998). Although these studies therefore serve to highlight the therapeutic promise of GnRH, it is essential that more research is performed in order to determine the extent to which these data are translatable into the treatment of human malignancies.

Extrapituitary Target Site	Putative Action	GnRH I/GnRH II	Reference
Placenta	Modulation of hCG release	GnRH I/GnRH II	(Islami et al., 2001)
T cell	Proliferation ↑	GnRH I analogue deslorelin	(Azad et al., 1997)
	Laminin receptor expression ↑	GnRH I/GnRH II	(Chen et al., 2002)
	Adhesion, chemotaxis and homing ↑	GnRH I/GnRH II	(Chen et al., 2002)
Ovarian granulosa-luteal cell	Progesterone secretion ↓	GnRH I analogue alarelin	(Kang et al., 2000)
Cytotrophoblast	uPA expression ↑	GnRH I/GnRH II	(Chou et al., 2002)
	PAI isoform 1 expression ↓	GnRH I/GnRH II	(Chou et al., 2002)
	MMP isoforms 2 and 9 expression ↑	GnRH I/GnRH II	(Chou et al., 2003c)
Decidual stromal cell	TIMP isoform 1 expression ↓	GnRH I/GnRH II	(Chou et al., 2003c)
	uPA expression ↑	GnRH I/GnRH II	(Chou et al., 2003a)
	PAI isoform 1 expression ↑	GnRH I	(Chou et al., 2003a)
	PAI isoform 1 expression ↓	GnRH II	(Chou et al., 2003a)
	MMP isoforms 2 and 9 expression ↑	GnRH I	(Chou et al., 2003b)
Olfactory neuron	Axon growth, cytoskeletal remodelling and migration ↑	GnRH I/GnRH I analogue buserelin	(Romanelli et al., 2004)
Sperm	Zona pellucida binding ↑	GnRH I	(Morales, 1998)
Melanoma cell	Proliferation, invasion and chemotaxis ↓	GnRH I analogue zoladex	(Moretti et al., 2002)
Gastric parietal cell	Gastric acid secretion ↓	GnRH I analogue alarelin	(Chen et al., 2005)

Table 1.6 Primary putative extrapituitary actions of GnRH I and GnRH II in humans. Table compiled from Cheng and Leung, 2005. Molecular biology of gonadotropin-releasing hormone (GnRH) I, GnRH II, and their receptors in humans. *Endocr Rev*, **26**, 283-306 and references shown above. hCG, human chorionic gonadotropin; uPA, urokinase type plasminogen activator; PAI, plasminogen activator inhibitor; MMP, Matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase. ↑, enhances function; ↓, reduces function.

1.3.2 GnRH Receptor

The GnRH receptor is a member of the rhodopsin subfamily of GPCRs (Tsutsumi et al., 1992, Stojilkovic et al., 1994) (figure 1.10). In general, the ECLs and the superficial regions of the TMs are involved in the binding of GnRH while the TMs are involved in conformational changes associated with receptor activation and signal propagation. The ICLs are thought to be involved in the interaction of the receptor with intracellular effectors. Although the mammalian type I receptor shares the common structural architecture of GPCRs within the rhodopsin subfamily, it is unique in that the carboxy-terminal tail is completely absent. In all other GPCRs, and both the non-mammalian GnRH receptors and the mammalian type II GnRH receptors, a carboxy-terminal tail is present and is a Ser/Thr phosphorylation target during receptor desensitisation. Willars and colleagues suggested that the lack of a carboxy-terminal tail in the rat receptor results in the inability of the receptor to undergo agonist-dependent phosphorylation and that this correlates directly with a resistance of the receptor to rapid desensitisation (Willars et al., 1999). Other studies have also demonstrated that the absence of a carboxy-terminal tail alters receptor trafficking dynamics. Indeed, a recent paper demonstrated that the catfish GnRH receptor, which possesses a carboxy-terminal tail, displays robust agonist-dependent internalisation (Pawson et al., 2008). In contrast, agonist stimulation of the human and the rat GnRH receptors does not significantly enhance internalisation above the observed constitutive level.

Despite the lack of a carboxy-terminal tail highlighted above, it may be possible that the YxxL (YFSL) motif at the intracellular end of TM7 in the mammalian type I GnRH receptor plays a role in GnRH-mediated signalling. The YFSL sequence has been suggested to be involved in the binding of PDZ domain containing scaffold proteins (Pawson and McNeilly, 2005). Similarly, studies indicate that the pYxxL motif forms part of the consensus sequence thought to be involved in the binding of SH2 domain containing Tyr phosphatases (SHPs) to immunoreceptors (Unkeless and Jin, 1997). Interestingly, however, there is only very limited evidence to suggest that the human GnRH receptor undergoes phosphorylation events (Liebow et al., 1991).

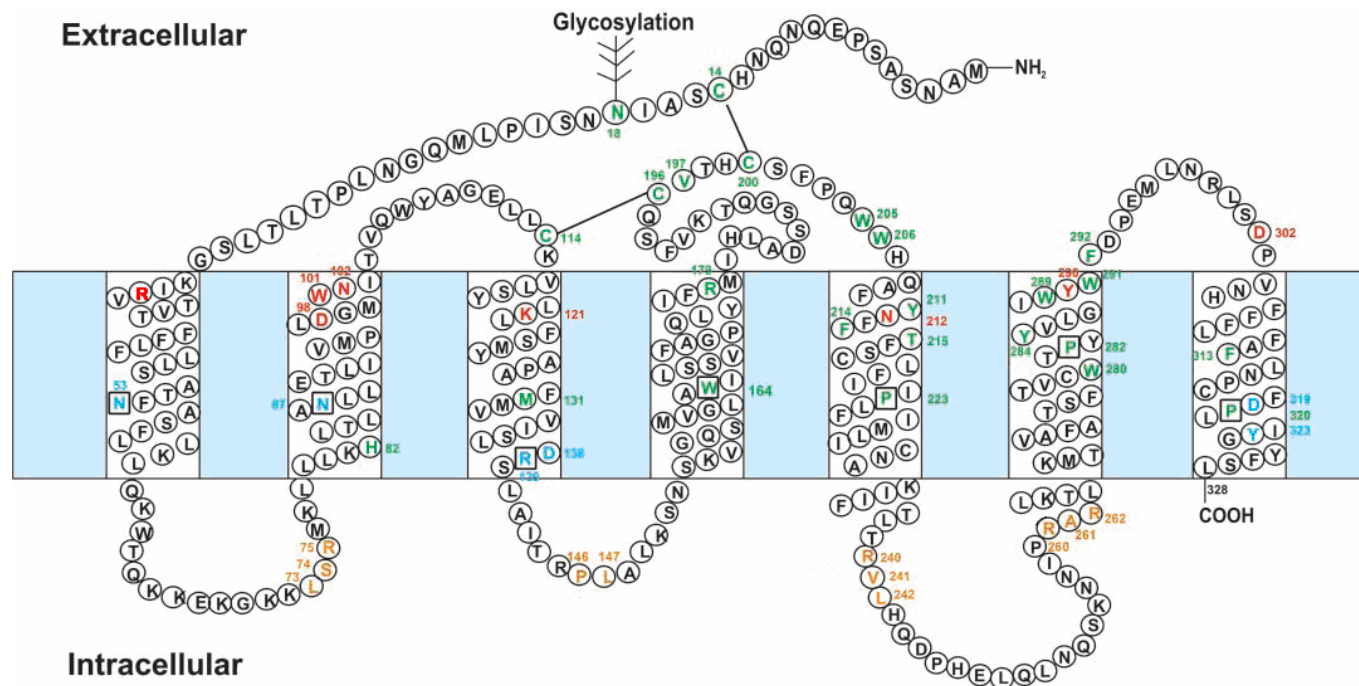


Figure 1.10 Two dimensional representation of the human GnRH receptor. The ECLs and the superficial regions of the TMs are involved in GnRH binding while the TMs (boxed) are involved in conformational changes associated with receptor activation and signal propagation. The ICLs are involved in interaction with both G proteins and non-G protein effectors. The receptor shares the common structural architecture of GPCRs within the rhodopsin subfamily. However, the carboxy-terminal tail is completely absent and there exists a reciprocal exchange of the conserved Asp⁸⁷-Asn³¹⁹ pair. Putative ligand binding sites and residues thought to be important in receptor structure or binding pocket formation are shown in red or green respectively. Residues thought to be involved in receptor activation are shown in blue. Residues in squares are highly conserved throughout rhodopsin subfamily GPCRs (see above). Residues thought to be involved in G protein coupling are shown in orange. Figure adapted from Millar et al., 2004. Gonadotropin-releasing hormone receptors. *Endocr Rev*, **25**, 235-275.

GnRH receptor expression has been reported both normally and pathologically in the pituitary, ovary (Oikawa et al., 1990, Kakar et al., 1994, Irmer et al., 1995, Fraser et al., 1996), uterus (Borri et al., 1998), prostate (Limonta et al., 1999), breast (Baumann et al., 1993, Kakar et al., 1994, Palmon et al., 1994, Mangia et al., 2002) and other non-reproductive tissues. Additionally, activation of the receptor has been suggested to regulate a plethora of biological effects (table 1.6 and (White et al., 2008b)). Given that both GnRH I and GnRH II can bind to the mammalian type I GnRH receptor with high affinity, taken together with the fact that the mammalian type II GnRH receptor is non-functional in man, it is frequently proposed that the pharmacological and signalling profiles of the human receptor differ, when compared to the gonadotrope, in extrapituitary tissues (Maudsley et al., 2004, Millar et al., 2004, Cheng and Leung, 2005, Lopez de Maturana et al., 2008, Millar et al., 2008). In the following sections, I will review both defined and contentious signalling pathways, the stimulation of which may be mediated by receptor activation.

1.3.3 GnRH Receptor-Mediated Signalling

Binding of GnRH to the GnRH receptor induces receptor activation and signal propagation. This results from the GnRH-mediated formation of new intermolecular and intramolecular interactions and a subsequent conformational change (Lu et al., 2007). Such a change is thought to uncover previously masked residues involved in effector coupling. Normal and pathological GnRH receptor signalling has been extensively reviewed (Stojilkovic et al., 1994, Kaiser et al., 1997, Kraus et al., 2001, Grundker et al., 2002, Grundker and Emons, 2003, Enomoto and Park, 2004, Harrison et al., 2004, Cheng and Leung, 2005, Dobkin-Bekman et al., 2006, Millar et al., 2008, White et al., 2008b) and, in order to retain focus, only the downstream regulation of the MAPK cascades and the gonadotropins will be discussed here. In addition, the G protein coupling ability of the receptor will later be examined.

1.3.3.1 MAPK

The intracellular transmission of extracellular signals is often mediated by several sets of MAPK cascades (Naor et al., 2000, Cuevas et al., 2007). They consist of tiers of protein kinases which sequentially activate each other by phosphorylation and regulate critical signalling pathways involved in the mediation of cellular proliferation, differentiation and apoptosis (Dobkin-Bekman et al., 2006, Goldsmith and Dhanasekaran, 2007). Upon activation, MAPKs translocate from the cytoplasm to the nucleus where they control the activity of various transcription factors by phosphorylation.

Much of the work presented within this thesis has involved studying the activation profiles and downstream effects of ERK, jun-N-terminal kinase (JNK) and P38 MAPK, three prominent members of the MAPK family. ERK activation by GnRH has been examined extensively. I and others have shown that ERK1/2 is markedly activated upon GnRH stimulation of GnRH receptor expressing cell lines (Sim et al., 1993, Sim et al., 1995, Reiss et al., 1997, Haisenleder et al., 1998, Morgan et al., 2008, White et al., 2008a). Inhibition of this pathway with PD98059 has been reported to significantly inhibit α glycoprotein subunit (α GSU) and FSH β gene transcription in rat gonadotrope cells (Haisenleder et al., 1998). Additionally, ERK activation has been studied with reference to the antiproliferative effects of GnRH and GnRH analogues. In this case, inhibition of the ERK cascade has been shown to be sufficient to inhibit such a GnRH analogue- (leuprolide) mediated effect (Kimura et al., 1999). Indeed, PD98059, a MEK1/2 inhibitor, was demonstrated to inhibit the leuprolide-induced dephosphorylation of the retinoblastoma protein, the hyperphosphorylation of which is a hallmark of G₁-S transition in the cell cycle.

In 2004, Davidson and colleagues described GnRH receptor-mediated signalling to ERK in HEK293 cells (Davidson et al., 2004). The activation of ERK was proposed to be independent of the activation of either PLC β or PKC. Instead, it was suggested to be dependent on protein-protein complex interactions between ERK, focal adhesion kinase (FAK) and src at focal adhesion complexes. The induction of the formation of this signalling complex assembly was shown to be mediated by the

GnRH receptor-induced activation of Rac. In contrast, in DU145 cells, GnRH-induced activation of ERK has been proposed to involve src and transactivation of the EGF receptor (Kraus et al., 2004) and, in α T31 cells, ERK activation by GnRH has been suggested to be wholly dependent on the activation of PKC (Benard et al., 2001) or both PKC and $G_{i/o}$ (Sim et al., 1995). These data indicate that the mechanism by which the GnRH receptor activates ERK may depend on the cell context in which experiments are carried out. Interestingly, within these different cellular backgrounds, the G proteins to which the GnRH receptor couples is the subject of much debate.

As with ERK, GnRH stimulates the activity of the JNK and the P38 MAPK pathways and Bonfil and colleagues showed that both MAPKs are involved in the GnRH-mediated induction of the ovine FSH β promoter in L β T2 gonadotrope cells (Bonfil et al., 2004). Activation of JNK has been reported to be highly dependent on the sequential activation of PKC, src and Rac/cdc42 in α T31 cells (Levi et al., 1998). P38 MAPK activation has also been shown to be reliant on PKC activation in the same cell type (Roberson et al., 1999). Both JNK and P38 MAPK have additionally been reported to be involved in promulgating the antiproliferative effects of GnRH. Maudsley and colleagues implicated JNK and P38 MAPK in the attenuation of cell growth in response to GnRH in JEG3 and BPH1 cells respectively (Maudsley et al., 2004) and Kraus and colleagues suggested that JNK activation may play a role in the apoptotic effect of GnRH in DU145 and PC3 cells (Kraus et al., 2004).

1.3.3.2 Gonadotropins

LH and FSH are heterodimeric glycoprotein hormones composed of the common α GSU bound to a specific β subunit (LH β and FSH β respectively) (Cheng and Leung, 2005). While both LH and FSH are released from the gonadotrope in a GnRH-independent constitutive manner, the pulsatile release of LH is totally dependent on a pulsatile GnRH input (Pawson and McNeilly, 2005). Although there is no clear association between pulsatile GnRH stimulation and the release of FSH, it is clear that GnRH does influence the secretion of this gonadotropin. Specific inhibition of GnRH has been shown to reduce the plasma FSH concentration in ewes

and this reduction is associated with a decrease in pituitary FSH β mRNA levels (McNeilly et al., 1991).

Regulated LH β gene transcription requires combinations of regulatory elements that cluster in the proximal and distal regions of the promoter (Salisbury et al., 2007). The proximal promoter region is conserved across all mammals and contains a pituitary homeobox 1 (PITX1) binding element flanked by regions that bind steroidogenic factor 1 (SF1) and early growth response 1 (EGR1). GnRH stimulates LH β gene expression by the transcriptional regulation of EGR1. EGR1 binds to and activates the LH β promoter by acting in combination with SF1 (Duan et al., 2002). GnRH and activin independently and synergistically activate FSH β transcription (Coss et al., 2007). FSH β gene expression is the limiting factor in FSH synthesis and this synthesis is the rate limiting step in FSH production. The molecular mechanisms underlying the transcriptional activity of this gene are not thus far fully understood. The rodent FSH β promoter has been shown to bind, and become activated by, SF1, Lim homeodomain transcription factor 3 (LHX3), nuclear factor Y (NFY), PITX1, PITX2, activator protein 1 (AP1) and Smad proteins (Melamed et al., 2006). The role of these proteins, specifically in GnRH-mediated FSH β transcriptional activity, has, however, not been well reported.

1.3.4 GnRH Receptor-G Protein Interaction

Historically, it was generally proposed that a given GPCR always interacted with a particular G protein or with multiple G protein subunits within one family (Hermans, 2003). However, for several GPCRs, it is now accepted that simultaneous functional coupling with G proteins of different families can be observed and that this coupling leads to the modulation of multiple intracellular effectors. Moreover, different ligands with distinct structures can induce differing changes in receptor conformation and, in doing so, alter the activity of different G protein subunits. For example, as reviewed in preceding sections, a group of ligands (DOI and LSD) with a similar ability to activate G_{q/11} by binding to the serotonin 5-HT_{2C} receptor display marked differences in their ability to activate G_{i3} (Cussac et al., 2002).

In pituitary gonadotropes, GnRH stimulates the biosynthesis and the secretion of LH and FSH by stimulating the GnRH receptor-mediated activation of the $G_{q/11}$ G proteins (Millar et al., 2008). However, the extrapituitary actions of GnRH in the nervous system, as well as in extraneural and neoplastic tissues, have been suggested to be modulated by pharmacologically dissimilar mechanisms to those regulating the effects in the pituitary. Specifically, with reference to the GnRH receptor-mediated inhibition of cellular proliferation and the induction of proapoptotic signalling mechanisms in cancer cells, it has been frequently suggested that these actions are mediated via the GnRH receptor-induced activation of the $G_{i/o}$ G proteins (Imai et al., 1996, Imai et al., 1997, Limonta et al., 1999, Grundker et al., 2001, Maudsley et al., 2004, Imai et al., 2006, Millar et al., 2008). Considering the variety of cellular effects influenced by GnRH, it seems plausible to postulate that coupling of the GnRH receptor to multiple G proteins would enable the alteration of the activity of multiple signal transduction pathways. However, as shall be discussed, this hypothesis is not uncontested (Grosse et al., 2000, White et al., 2008a).

1.3.4.1 Experimental Determination of GPCR-G Protein Interaction

Several experimental strategies have been employed to analyse the selectivity of GPCR-G protein interaction. One frequently used approach is to study the change in a biochemical response mediated by the stimulation of a given GPCR in the absence or the presence of chemical inhibitors. While these studies provide useful preliminary assays to evaluate the utilisation of specific G proteins by different GPCRs, they do not provide direct evidence of the interaction of a given GPCR with a specific G protein. Indeed, as mentioned, PTX may specifically interfere with the coupling of receptors to most members of the $G_{i/o}$ family (with the exception of G_z). As such, the PTX sensitivity of an activated GPCR-induced signal is frequently proposed to provide an indication of GPCR- $G_{i/o}$ interaction (Ruf et al., 2003). However, the observation by Shan and colleagues of RTK-induced G protein subunit activation (Shan et al., 2006), taken together with the knowledge that GPCRs themselves may transactivate RTKs (Delcourt et al., 2007), dictates that such sensitivity does not provide direct evidence of GPCR- $G_{i/o}$ coupling. Similarly, cholera toxin (CTX) may be used to pharmacologically modulate the G proteins of

the G_s family (Milligan and Kostenis, 2006). CTX application results in the ADP ribosylation of the $G\alpha_s$ subunits, the subsequent persistent activity of the adenylyl cyclases and thus the sustained elevation of intracellular cAMP. Given that it has been demonstrated that the $G\beta\gamma$ complex (Gao and Gilman, 1991, Tang and Gilman, 1991, Yoshimura et al., 1996, Steiner et al., 2006) is also able to activate adenylyl cyclase enzymes, it is not simply sufficient to surmise direct GPCR- G_s interaction from studies which observe similar signalling outputs with CTX as with stimulation of a given receptor.

Clearly, therefore, the determination of direct GPCR-G protein interaction must rely on more than the simple analysis of positive second messenger responses to chemical inhibitors. There are a number of ways by which this can be achieved. For example, specific G protein antibodies have been successfully used to study the specificity of direct receptor-G protein interaction with the scintillation proximity assay (DeLapp et al., 1999). Antibodies raised against specific G protein subunits or epitope tags have also been used to demonstrate such interaction by coimmunoprecipitation (Lachance et al., 1999, Feng et al., 2002). A more recent approach involves using cell lines deficient in specific G protein subunits to study ligand-induced signalling (Riobo and Manning, 2005). This method has the inherent advantage of maintaining an intact cell environment but is generally constrained to MEF cells. In this thesis, I have utilised the latter two methodologies discussed.

1.3.4.2 GnRH Receptor Activation of the Heterotrimeric G Proteins

Activation of multiple heterotrimeric G proteins by the agonist-bound GnRH receptor is the subject of much controversy. Numerous studies infer a direct interaction between this GPCR and G proteins of different families (table 1.7 and references therein). However, debatably erroneous conclusions, based on indirect second messenger outputs in response to toxin treatments, form the vast majority of the published data to date. For example, GnRH analogue- (leuprolide) induced ERK activation has been shown to be sensitive to PTX suggesting the involvement of $G_{i/o}$ in the GnRH-mediated stimulation of ERK (Kimura et al., 1999). In 2000, Grosse and colleagues attempted to directly address this issue by using a non-hydrolysable

GTP analogue, [α - 32 P]GTP azidoanilide, and subsequent immunoprecipitation with antisera raised against specific G α subunits (Grosse et al., 2000). Their investigations revealed exclusive GnRH-induced photolabelling of G $\alpha_{q/11}$ in α T31 and CHO membrane preparations suggesting that the GnRH receptor does not interact directly with G $_{i/o}$. These studies emphasise the unresolved nature of the G protein coupling selectivity of the mammalian GnRH receptor, a critical issue which must be addressed in detail before the full potential therapeutic value of GnRH may be exploited.

G Protein Family/Families	Assay(s) Used	Cell Type(s)	Reference
G _{i/o}	PTX	αT31	(Sim et al., 1995)
G _{i/o}	PTX	Reproductive tract tumours	(Imai et al., 1996)
G _{i/o}	MAPK activity, PTX	Caov3	(Kimura et al., 1999)
G _{i/o}	cAMP production, PTX	DU145, LNCaP	(Limonta et al., 1999)
G _{i/o}	cAMP production, PTX	BPH1, JEG3	(Maudsley et al., 2004)
G _{i/o}	PTX	Caov3, SKOv3	(Imai et al., 2006)
G _{i/o} , G _s	Inositol phosphate production, PTX, CTX	Pituitary	(Hawes et al., 1993)
G _{i/o} , G _s	cAMP production, PTX	FNCB4	(Romanelli et al., 2004)
G _s	PTX, CTX	Pituitary	(Janovick and Conn, 1993)
G _s	cAMP production, CTX	GGH ₃	(Kuphal et al., 1994)
G _s	cAMP production	GGH ₃	(Stanislaus et al., 1996)
G _s	cAMP production, GnRH receptor gene transcription	GGH ₃	(Lin and Conn, 1998)
G _s	cAMP production	Ovarian tumours	(Chamson-Reig et al., 2003)
Not G _s	cAMP production, CTX	Pituitary	(Conn et al., 1979)
G _{q/11} , G _s	Inositol phosphate production, cAMP production	GGH ₃	(Awara et al., 1996)
G _{q/11} , G _s	Inositol phosphate production, cAMP production	Sf9	(Delahaye et al., 1997)
G _{q/11} , G _s	Inositol phosphate production, cAMP production	COS7	(Arora et al., 1998)
G _{q/11} , G _s	Inositol phosphate production, cAMP production	GGH ₃	(Ulloa-Aguirre et al., 1998)
G _{q/11} , G _s	MAPK activity, PTX, CTX	GGH ₃	(Han and Conn, 1999)

G _{q/11} , G _s	PTX, peptide-induced uncoupling of G protein subunits from GPCR, G protein α subunit protection from trypsin digestion	L β T2	(Liu et al., 2002b)
G _{q/11} , G _s	Inositol phosphate production, cAMP production	CV1	(Oh et al., 2003)
G _{q/11} , G _s , G _{i/o}	Inositol phosphate production, PTX, CTX	Pituitary	(Barnes and Conn, 1993)
G _{q/11} , G _s , G _{i/o}	Inositol phosphate production, PTX, CTX	GGH ₃	(Janovick and Conn, 1994)
G _{q/11} , G _s , G _{i/o}	GnRH-induced palmitoylation, cAMP production, inositol phosphate production	Pituitary, GGH ₃	(Stanislaus et al., 1998b)
G _{q/11} , G _s , G _{i/o}	cAMP production, Ca ²⁺ production, inositol phosphate production, membrane-bound G α subunit release	GT17	(Krsmanovic et al., 2003)
G _{q/11} , G _s , G _{i/o}	cAMP production, inositol phosphate production, PTX, CTX	CHO, COS7, GH ₃ , HEK293, HeLa	(Knollman and Conn, 2008)
G _{q/11}	DAG production	Gonadotrope	(Andrews and Conn, 1986)
G _{q/11}	Inositol phosphate production	α T31	(Hsieh and Martin, 1992)
G _{q/11}	GnRH enhanced G protein α subunit downregulation	α T31	(Shah and Milligan, 1994)

G _{q/11}	GnRH enhanced G protein α subunit downregulation	α T31	(Shah et al., 1995)
G _{q/11}	Inositol phosphate production, DAG production	α T31	(Poulin et al., 1996)
G _{q/11}	PTX	α T31	(Reiss et al., 1997)
G _{q/11}	GnRH-induced palmitoylation, inositol phosphate production, GnRH enhanced G protein α subunit downregulation	Pituitary, GGH ₃	(Stanislaus et al., 1997)
G _{q/11}	Redistribution of G α subunits	Pituitary	(Cornea et al., 1998)
G _{q/11}	LH release, steroid production	G α subunit knockout mice	(Stanislaus et al., 1998a)
G _{q/11}	Inositol phosphate production, cAMP production, CRE transcription, PTX, GTP photolabelling	α T31, CHO, COS7	(Grosse et al., 2000)
G _{q/11}	MAPK activity, GTP γ S binding, cAMP production	G α _{q/11} knockout MEF, SCL60	(White et al., 2008a)

Table 1.7 Studies of the G protein coupling profile of the GnRH receptor. Table compiled from Ruf et al., 2003. Structure of the GnRH receptor stimulated signalling network: insights from genomics. *Front Neuroendocrinol*, **24**, 181-199 and references shown above.

1.3.4.3 Molecular Basis of GPCR-G Protein Coupling Specificity

Over the past two decades, the G protein coupling profile of many GPCRs has been elucidated in considerable detail using various experimental approaches. One largely unsuccessful method used to predict the G protein coupling specificity of a given receptor was to analyse the primary sequence of the protein (Wess, 1998). However, the amino acids predicted to determine specific coupling properties are generally not well conserved among other receptors that display a similar coupling profile (Wong, 2003). Thus, a conserved G protein coupling motif, which directs signalling selectivity at given receptors, is not thought to exist. Instead, evidence suggests that such selectivity is dictated by the conformation of the intracellular regions of the receptor. This hypothesis accounts for the observation that different carboxy-terminal splice variants of the prostaglandin EP₃ receptor exhibit different G protein coupling profiles (Namba et al., 1993). Furthermore, it is supported by the demonstration that the mutation of Lys⁵⁸³ in ECL3 of the rat LH receptor to Ala, Arg, Glu and Leu completely disrupts G_s coupling (Gilchrist et al., 1996). A slight change in a particular receptor conformation may therefore alter the selectivity of G protein coupling for a given GPCR and, thus, the efficacy of signalling at a given pathway.

Experimental evidence has shown that ICL2 and ICL3 are thought to be involved in determining the selectivity of interaction between a GPCR and G proteins as well as the efficiency of G protein activation. The exchange of ICL3 between different chemokine receptors revealed that this loop largely determines G_q coupling selectivity (Arai and Charo, 1996). Additionally, mRNA editing of the serotonin 5-HT_{2C} receptor results in receptor isoforms which differ in their ICL2 sequences and vary considerably in their G_q coupling efficiencies (Burns et al., 1997). Similarly, evidence suggests that ICL1 may also regulate receptor-G protein coupling selectivity. Indeed, Wu and colleagues showed that an amino acid sequence derived from ICL1 of the CCK_A receptor is able to confer the ability to couple to G_s upon the CCK_B receptor (Wu et al., 1997). In the case of the GnRH receptor, it has been suggested that ICL1 is involved in the activation of G_s (Arora et al., 1998) while ICL2 and ICL3 are important in mediating the activation of G_{q/11} (Millar et al.,

2004). The receptor domains thought to be important in the coupling of the receptor to $G_{i/o}$ remain undefined.

Although receptor conformation undoubtedly plays a role in defining the selectivity of receptor-G protein interaction, it is not the exclusive determinant. Receptor phosphorylation and palmitoylation, interaction of a GPCR with accessory proteins, the cellular expression levels of different G protein subunits and the G protein structure itself may all contribute to the mediation of such selectivity (Wess, 1998, Millar et al., 2004, Morfis et al., 2008). Evidence indicates that the carboxy-terminal portions of the $G\alpha$ subunit play a key role in dictating the specificity of receptor-G protein coupling (Conklin et al., 1993). In addition, the amino-terminus of the $G\alpha$ subunit and the composition of the $G\beta\gamma$ complex may also participate in coupling determination. Indeed, Figler and colleagues showed that, when the A_1 adenosine receptor was reconstituted with G protein $\beta\gamma$ complexes of defined subunit composition, the $G\beta_1/G\gamma_1$ complex is markedly less effective than either the $G\beta_1/G\gamma_2$ or the $G\beta_1/G\gamma_3$ complex in promoting receptor-G protein coupling (Figler et al., 1997).

1.3.5 Summary

Numerous studies infer that the GnRH receptor may have the inherent ability to couple to and activate multiple G proteins. Additionally, other reports postulate exclusive interaction of the agonist-bound receptor with G proteins of the $G_{q/11}$ family (Grosse et al., 2000). When viewed collectively with the potential therapeutic value of GnRH, these studies serve to underscore the necessity to fully evaluate the intrinsic G protein coupling ability of this GPCR.

1.4 Outline of Thesis

A desire to finally delineate the intrinsic G protein coupling ability of the activated GnRH receptor, in an intact cell environment, represents the underlying motivation behind the studies that make up this thesis. This aspiration stems from my interest in defining the mechanism by which GnRH may inhibit the proliferation of reproductive cancer cells.

In chapter three I provide, in my opinion, convincing evidence to suggest that the GnRH receptor does not possess the ability to interact directly with the G proteins of the $G_{i/o}$ or the G_s families. Additionally, I show that $G_{q/11}$ is critical for the induction of antiproliferative signalling by GnRH I but that the signalling mechanism involved in the stimulation of such an effect is dissimilar to the canonical signalling pathway activated by this G protein family. Instead, I implicate src Tyr kinase and the ERK, but neither the JNK nor the P38 MAPKs, in the induction of the GnRH I-mediated inhibition of cell growth.

In chapter four, based on my previous observations, I identify a novel GnRH receptor- $G_{12/13}$ -mediated signalling pathway. Initially, I use a variety of second messenger systems to associate this signal transduction cascade with GnRH-mediated signalling. However, I then confirm, in an intact cell system, that the $G_{12/13}$ G protein family may interact directly with this GPCR. Furthermore, I show that GnRH I and GnRH II can induce distinct and dramatic changes in the actin cytoskeleton (which are indicative of the differential activation of the Rac and the Rho monomeric G proteins). Based on my experimental data and an abundance of published literature, I suggest that these changes reflect the activation of both this newly identified signalling cascade and a recently discovered GnRH receptor-mediated G protein-independent src-induced pathway. Indeed, I go on to provide evidence to confirm that the GnRH receptor may interact with src and that GnRH I but not GnRH II may, independently of $G_{q/11}$, activate this Tyr kinase. Finally, I propose the involvement of the $G_{12/13}$ G protein family but not src in the transcriptional regulation of LH β and FSH β in the pituitary gonadotrope.

Both of the results chapters within this thesis are designed to read independently. They contain a short abstract and introduction in an effort to familiarise the reader with the details relevant to the appropriate aspect of GPCR-/GnRH receptor-mediated signalling. However, it is hoped that, when all the included data is viewed collectively, a cohesive and accurate description of GnRH receptor-G protein interaction and the subsequent signalling associated with such cellular communication will be attained.

Chapter Two

Materials and Methods

2.0 Introduction

This chapter details the materials and the laboratory techniques used in the completion of the research presented within this thesis. All materials used that were obtained from external sources and any work not performed by myself has been acknowledged. Any reagents with no stated manufacturer were obtained from Sigma (Dorset, UK).

2.1 Materials

The pMEP4 expression vector was kindly provided by Dr. Keith Leppard, University of Warwick, UK. The MEK1/2 inhibitor PD98059, the JNK inhibitor SP600125, the P38 inhibitor SB203580, the PKC inhibitor Ro-32-0432, the Ca^{2+} chelator BAPTA-AM, the src Tyr kinase inhibitor PP2 and the PLC β inhibitor U-73122 were all obtained from Calbiochem (Nottingham, UK). YM-254890 was kindly provided by Dr. Masatoshi Taniguchi, Astellas Pharma, Japan. The $\text{G}\alpha_{q/11}$, the $\text{G}\alpha_{i1}$, the $\text{G}\alpha_{i2}$, the $\text{G}\alpha_{i3}$, the $\text{G}\alpha_{12}$, the $\text{G}\alpha_{13}$, the $\text{G}\alpha_q\text{Q209L}$, the $\text{G}\alpha_{i2}\text{Q205L}$, the $\text{G}\alpha_s\text{Q227L}$, the $\text{G}\alpha_{12}\text{Q231L}$, the $\text{G}\alpha_{13}\text{Q226L}$ and the $\text{G}\alpha_{i2}\text{G203T}$ cDNAs were all obtained from the cDNA Resource Centre (Missouri, USA). The $\text{G}\alpha_{13}\text{G225A}$ cDNA was kindly provided by Dr. Stefan Offermanns, University of Heidelberg, Germany and the $\Delta\text{DH-p115}$ RhoGEF cDNA was kindly provided by Dr. Philip Wedegaertner, Kimmel Cancer Centre, USA. The SRE-luciferase reporter cDNA was kindly provided by Dr. Adam Pawson, MRC Human Reproductive Sciences Unit, UK. The LH β luciferase reporter and the FSH β luciferase reporter cDNAs and the recombinant adenovirus expressing green fluorescent protein (GFP) were all kindly provided by Dr. Pamela Brown, MRC Human Reproductive Sciences Unit, UK. The $\text{G}\alpha_{q15}$ cDNA and the $\text{G}\alpha_{q/11}$ knockout MEF cell line were both kindly provided by Professor Graeme Milligan, University of Glasgow, UK. The $\text{G}\alpha_{q/11}$ knockout MEF cell line was originally derived from a combined $\text{G}\alpha_{q/11}$ double knockout mouse and has been previously shown to have absolutely no endogenous $\text{G}\alpha_{q/11}$ (Offermanns et al., 1998, Stevens et al., 2001). The L β T2 cell line was kindly provided by Dr. Pamela Mellon, University of California, USA. The recombinant adenovirus expressing $\text{G}\alpha_q$ was kindly provided by Dr. Nicholas Webster, University of California, USA. The pERK1/2, the pJNK, the pP38, the ERK1/2, the psrc and the

src antibodies were all obtained from Cell Signalling Technology (Hertfordshire, UK). The $G\alpha_{q/11}$, the $G\alpha_{i/o}$, the $G\alpha_{13}$, the HA agarose conjugate and the PY20 agarose conjugate antibodies were all obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The HA and the β -actin antibodies were both obtained from Abcam (Cambridge, UK). Activin A was obtained from R&D Systems (Minneapolis, USA).

2.2 Transformation of Competent Cells

XL-10 Gold competent cells (Stratagene, Cheadle, UK) were thawed on ice and incubated with 1.5 μ l β -mercaptoethanol for 10 minutes. 10 ng plasmid DNA was added and the bacteria left on ice for an additional 30 minutes. After incubation, the cells were heat shocked at 42 °C for 35 seconds and immediately placed back on ice. 450 μ l S.O.C. medium (Invitrogen, Paisley, UK) was added and the bacteria shaken at 37 °C for 1 hour prior to being streaked out on a Luria Broth- (LB) agar plate (containing 100 μ g/ml ampicillin) and incubated at 37 °C overnight.

2.3 Preparation of Plasmid DNA

A single colony transformed bacteria was picked and used to inoculate 5 ml LB medium containing 100 μ g/ml ampicillin. The culture was grown at 37 °C overnight with constant shaking. The following evening, 1 ml was used to inoculate 250 ml fresh LB medium (containing 100 μ g/ml ampicillin) and the culture grown for a further 16 hours. Plasmid DNA was purified using Qiagen maxi-preparation columns (Qiagen, Crawley, UK) according to the manufacturers instructions and eluted in TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA). The DNA was examined by agarose gel electrophoresis and the concentration and the quality were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Delaware, USA).

2.4 Preparation of Glycerol Stocks

Glycerol stocks were made by adding 300 μ l sterile 80% (v/v) glycerol to 700 μ l bacterial culture that had been grown overnight. Vials were inverted to mix the glycerol, frozen on dry ice and stored at -80 °C. To recover the bacteria, a sterile inoculating loop was used to scrape the surface of the frozen culture. The bacteria

were streaked out on a LB-agar plate (containing 100 µg/ml ampicillin) and grown at 37 °C overnight. A single colony was picked and plasmid DNA prepared as described.

2.5 Agarose Gel Electrophoresis

1% (w/v) agarose gels were prepared in TAE buffer (40 mM Tris, 320 mM acetic acid, 1 mM EDTA, pH 7.2). Plasmid DNA and restriction digests were separated at 120 V for 40 minutes, stained with ethidium bromide and visualised under ultraviolet light using a GeneFlash transilluminator (Syngene Bio Imaging, Cambridge, UK). For preparative work, a 366 nm light source was used in order to minimise photonic nicking and dimerisation of DNA. The DNA was recovered from agarose gel slices using a QIAquick gel extraction kit (Qiagen, Crawley, UK) according to the manufacturers instructions.

2.6 Restriction Digests

Double restriction digests of plasmid DNA were performed in a 20 µl final volume using 1 unit appropriate enzymes (Promega Madison, USA) in a buffer which was chosen to give maximum enzymatic activity. Digestions were performed at 37 °C for between 1 and 3 hours. DNA was fractionated by agarose gel electrophoresis and the required products were recovered as described.

2.7 Ligation of DNA

Inserts were ligated into linearised vectors with cohesive ends using T4 DNA ligase (Promega Madison, USA). 100 ng vector and an appropriate mass insert were made up to 8 µl using sterile H₂O. 1 µl 10 x T4 DNA ligase buffer (300 mM Tris (pH 7.8), 100 mM MgCl₂, 100 mM DTT, 100 mM ATP) and 1 µl T4 DNA ligase were added and the reaction allowed to proceed at 12 °C for 16 hours. Control reactions were set up with either vector alone or insert alone. 1 µl ligation mixture was used to transform XL-10 Gold competent cells and the transformants were selected on LB-agar plates (containing 100 µg/ml ampicillin) as described. Single colonies were picked and used to inoculate 5 ml LB medium (containing 100 µg/ml ampicillin) and the culture was grown at 37 °C overnight with constant shaking. The bacteria were

harvested by centrifugation at 6,000 x *g* for 5 minutes and plasmid DNA prepared using Qiagen mini-preparation columns (Qiagen, Crawley, UK) according to the manufacturers instructions. Restriction digests were performed as described to confirm the presence of DNA inserts and the plasmids sequenced to ensure the absence of mutations. Dideoxy sequencing reactions were carried out at the MRC Human Genetics Unit, Edinburgh, UK.

2.8 Cell Culture

Full length GnRH receptor cDNA was cloned into pMEP4 at *Not*I and *Xho*I and the construct transfected into $G\alpha_{q/11}$ knockout MEF cells by electroporation. Cloned cells were selected using hygromycin resistance and screened by radioligand binding. $G\alpha_{q/11}$ knockout MEF cells stably expressing the GnRH receptor were maintained in Dulbeccos Modified Eagles medium (DMEM) supplemented with 10% (v/v) foetal calf serum (PAA Laboratories, Somerset, UK), 2 mM glutamine, 50 IU/ml penicillin, 50 IU/ml streptomycin, 0.5 mg/ml G418 sulphate and 50 µg/ml hygromycin (Invitrogen Life Technologies, Paisley, UK) at 37 °C in a humidified 5% CO₂ atmosphere. HEK293 cells stably expressing the GnRH receptor (designated SCL60) and BHK cells stably expressing the GnRH receptor and an SRE-luciferase reporter were generated previously within our laboratory. Both cell lines were maintained in DMEM supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, 50 IU/ml streptomycin and 0.5 mg/ml G418 sulphate at 37 °C in a humidified 5% CO₂ atmosphere. COS7 cells and LβT2 cells were maintained in DMEM supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 50 IU/ml penicillin and 50 IU/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. LβT2 cells were routinely grown on matrigel (BD Biosciences, New Jersey, USA) coated cell culture vessels.

All cell lines were routinely passaged twice weekly by enzymatic dispersal with trypsin. Briefly, the medium was removed from confluent cultures and the cells washed once in phosphate buffered saline (PBS). 2 ml trypsin was added to each 162 cm² cell culture flask (Fisher Scientific, Leicestershire, UK) and the flasks returned to the incubator for between 2 and 5 minutes. After successful dispersal, 8

ml growth medium was added in order to quench the trypsin. All cell lines were routinely split 1:3. Excess dispersed cells were diluted 1:10 in PBS and counted using a Neubauer haemocytometer. All four counting areas were utilised and an average taken to give the cell number $\times 10^5/\text{ml}$. The correct volume cell suspension was then seeded onto cell culture dishes for experiments as appropriate.

2.9 Cryopreservation and Resuscitation of Immortalised Cell Lines

Stocks of each cell line were stored at $-196\text{ }^{\circ}\text{C}$ under liquid nitrogen in cryoprotectant (10% (v/v) dimethylsulphoxide (DMSO), 90% (v/v) foetal calf serum). Cells were recovered from the liquid nitrogen store and warmed rapidly to $37\text{ }^{\circ}\text{C}$ prior to being gently resuspended in growth medium and seeded into cell culture flasks. Frozen stocks were preserved by banking the cells for the first two passages after they were resuscitated. Confluent cultures were passaged as described and the cells collected by centrifugation at $500 \times g$ for 3 minutes. The growth medium was decanted and the cell pellet gently resuspended in cryoprotectant, aliquoted into vials and frozen at $-80\text{ }^{\circ}\text{C}$ in a cryo $1\text{ }^{\circ}\text{C}$ freezing container (Nalgene, Hereford, UK). After 24 hours, the vials were transferred to liquid nitrogen for long term storage.

2.10 Transient Transfection by Adenoviral Infection

$G\alpha_{q/11}$ knockout MEF cells were transfected (for sulforhodamine B (SRB) and trypan blue dye exclusion assays only) using a recombinant adenovirus expressing $G\alpha_q$ essentially as previously described (Liu et al., 2005). Briefly, 0.5×10^5 cells/ml were seeded onto 12 well cell culture dishes (1 ml/well) and allowed to attach overnight. The following day, the growth medium was removed and the cells were infected at a multiplicity of infection (MOI) of 90 plaque forming units (PFU)/cell for 16 hours. A recombinant adenovirus expressing GFP was used as a control. To allow protein expression, the infected cells were left for 48 hours prior to the commencement of experiments.

2.11 Transient Transfection by Electroporation

COS7 cells and $G\alpha_{q/11}$ knockout MEF cells were transfected by electroporation essentially as previously described (Lu et al., 2005, Lu et al., 2007). Briefly, 1.0×10^5 cells/ml were seeded onto 162 cm² cell culture flasks (25 ml/flask) and allowed to grow for 96 hours prior to transfection. The cells within each cell culture flask were enzymatically dispersed as described and resuspended in 0.7 ml 4 °C Optimem (Invitrogen Life Technologies, Paisley, UK). Transient transfections were performed by electroporation, using a Bio-Rad Gene Pulser XCell (Bio-Rad Laboratories, Hertfordshire, UK), at 230 V/950 μ F for COS7 cells and 320 V/500 μ F for $G\alpha_{q/11}$ knockout MEF cells with 20 μ g plasmid DNA and 0.7 ml cell suspension in each 0.4 cm electroporation cuvette. After 10 minutes, the contents of each electroporation cuvette were resuspended in growth medium and seeded onto cell culture dishes for experiments as appropriate.

2.12 Transient Transfection with Fugene

L β T2 cells and BHK cells were transfected with Fugene (Roche, Welwyn Garden City, UK) using 6 μ l Fugene/1 μ g plasmid DNA. 6×10^5 cells/ml were seeded onto 12 well cell culture dishes (1 ml/well) and allowed to attach overnight. The following day, 12 μ l Fugene was added to 100 μ l 37 °C Optimem and the solution vortexed for 3 seconds. 5 minutes later, 2 μ g plasmid DNA was added and the solution vortexed, centrifuged briefly and left for 45 minutes to ensure transfection complex formation. 100 μ l transfection mix was added directly to the growth medium in each well and the dishes swirled to allow complete mixing. The cells were returned to the incubator and routinely harvested for experiments either 48 or 72 hours after transfection.

2.13 Preparation of Pharmacological Inhibitors and Ligands

Stock solutions of inhibitors were routinely prepared in DMSO as per the manufacturers instructions, aliquoted and stored at -20 °C. Stock solutions of GnRH I and GnRH II were prepared in 20% (v/v) propylene glycol, rapidly frozen in a cardice bath and stored at -20 °C. On the day of experiments, GnRH aliquots were defrosted and diluted in 20% (v/v) propylene glycol and, subsequently, growth

medium or buffer to the desired concentration. All inhibitors and ligands, except for U-73122, were subjected to a maximum of two freeze/thaw cycles. U-73122 was freshly prepared for each experiment.

2.14 Radioligand Binding Assay

Radioligand binding assays were performed on intact cells as previously described (Lu et al., 2005, Lu et al., 2007, Coetsee et al., 2008, Stewart et al., 2008, White et al., 2008a). Briefly, $G\alpha_{q/11}$ knockout MEF cells, L β T2 cells and SCL60 cells, on 12 well cell culture dishes, were incubated with ^{125}I -[His⁵,D-Tyr⁶]GnRH (100,000 cpm/0.5 ml/well) and either vehicle (0.2% (v/v) propylene glycol) or various concentrations of unlabelled GnRH in a radioligand binding assay buffer (0.1% (w/v) bovine serum albumin (BSA), 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), DMEM) for 4 hours at 4 °C. Non-specific binding was determined in the presence of 1 μM unlabelled GnRH. After incubation, the free ligand was removed with two rapid washes in cold PBS and the cells solubilised in 0.5 ml 0.1 M NaOH. Radioactivity was counted by γ -spectrometry. For GnRH receptor expression level comparison, cells that had been seeded in parallel were enzymatically dispersed and counted as described. Data is presented as counts/minute/million cells.

2.15 Preparation of Cellular Extracts

Appropriately transfected or untransfected $G\alpha_{q/11}$ knockout MEF cells, COS7 cells or SCL60 cells were seeded onto 100 mm cell culture dishes (at a density of 3×10^5 cells/ml with 10 ml/dish for SCL60 cells) and allowed to attach overnight. The growth medium was removed and the cells were washed twice in PBS prior to being incubated in serum free medium (DMEM supplemented with 2 mM glutamine, 50 IU/ml penicillin, 50 IU/ml streptomycin and 10 mM HEPES) for 16 hours in the presence or absence of chemical inhibitors as described in the figure legends. GnRH stimulations were performed at 37 °C in fresh serum free medium in the presence or absence of chemical inhibitors as described in the figure legends. Following appropriate GnRH stimulation, the cell monolayers were placed on ice, washed once in cold PBS and lysed in a Nonidet P-40 solubilisation buffer (250 mM NaCl, 50

mM HEPES, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, 2 mM EDTA, pH 8.0) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride and 10 µg/ml leupeptin. The solubilised lysates were clarified by centrifugation at 20,000 x *g* for 15 minutes and the nuclear contents were sheared by subsequent sonication. Sample protein concentrations were measured as described and the samples were diluted with sterile H₂O to a concentration of 1 mg/ml total protein.

2.16 Determination of Protein Concentration

Protein concentrations were determined using the modified Bradford assay (Bio-Rad Laboratories, Hertfordshire, UK). BSA was used as a standard. A standard curve (0 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 1.5 mg/ml and 2.0 mg/ml) was prepared by the serial dilution of a 10 mg/ml BSA stock in 200 µl Bradford dye and an appropriate volume PBS (such that the final volume was made up to 1 ml). 5 µl each sample was added to 795 µl PBS and 200 µl Bradford dye. All standards and samples were vortexed and left for 5 minutes. Absorbances were measured at 595 nm using a spectrophotometer and a standard curve plotted. The concentration of protein in each sample was calculated from the equation of the line.

2.17 Immunoprecipitation

Proteins were immunoprecipitated from 500 µg cellular extract with 20 µl appropriate agarose conjugated antibody slurry. Briefly, samples were tumbled at 4 °C overnight and immunoprecipitates collected by centrifugation at 20,000 x *g* for 5 minutes. Immune complexes were washed three times in 1 ml Nonidet P-40 solubilisation buffer supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride and 10 µg/ml leupeptin to remove contaminating proteins. To allow only specific binding of proteins to the agarose conjugate, cellular extracts were routinely cleared with 20 µl appropriate agarose conjugated antibody slurry for 2 hours prior to immunoprecipitating.

2.18 Western Blotting

Clarified cellular extracts were mixed with an equal volume 2 x Laemmli sample buffer (LSB) (80% (v/v) 10% (w/v) SDS, 5% (v/v) β -mercaptoethanol, 10% (v/v) glycerol, 2.5% (v/v) 1M Tris (pH 7.0), 2.5% (v/v) distilled H₂O), heated to 100 °C for 5 minutes and allowed to cool. Immunoprecipitates were routinely solubilised in 25 μ l LSB, heated to 100 °C for 5 minutes and allowed to cool. Agarose beads were pelleted by centrifugation at 20,000 x g for 1 minute. Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 20% Tris-Gly gels (Invitrogen Life Technologies, Paisley, UK). Electrophoretic separation was performed at 45 mA for 1 hour in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 1% (w/v) SDS).

After electrophoretic separation, proteins were electroblotted on to polyvinylidene difluoride (PVDF) membranes (NEN Life Sciences, Buckinghamshire, UK) for protein immunoblotting. PVDF membranes were prepared by washing in 100% methanol for 5 minutes followed by several 10 minute washes in distilled H₂O. Finally, they were equilibrated in semi-dry transfer buffer (20 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS) for at least 30 minutes. Six thicknesses electrode paper (Bio-Rad Laboratories, Hertfordshire, UK) were soaked in semi-dry transfer buffer for at least 10 minutes prior to electroblotting. Three thicknesses electrode paper were placed onto the anode plate of the transfer apparatus and the PVDF membrane placed on top. The Tris-Gly gel was next placed onto the stack and another three thicknesses electrode paper placed on top of it. Air bubbles were carefully rolled out with a glass test tube and the cathode plate placed on top. Proteins were electroblotted at 25 V for 1 hour.

Following electroblotting, the membranes were rinsed for 10 minutes with TBS-T (100 mM Tris (pH 7.0), 150 mM NaCl, 0.05% (v/v) Tween 20, 0.05% (v/v) Nonidet P-40) and blocked for at least 1 hour in 10 ml blocking buffer (4% (w/v) BSA, TBS-T) with gentle shaking. The blocking buffer was discarded and a primary antibody (diluted 1:1000 in 10 ml blocking buffer) applied at 4 °C overnight. Each primary antibody was used a maximum of 10 times before being replaced. PVDF membranes

were washed three times in TBS-T for 10 minutes after which an appropriate alkaline phosphatase conjugated polyclonal secondary antibody (diluted 1:10,000 in 10 ml blocking buffer) was applied for 1 hour. After three further 10 minute washes in TBS-T, the immunoblots were drained and developed with an enzyme linked chemifluorescence (ECF) substrate (GE Healthcare, Buckinghamshire, UK) according to the manufacturers instructions. Protein bands were visualised using a Typhoon 9200 PhosphorImager (GE Healthcare, Buckinghamshire, UK) and quantified with ImageQuant version TL (GE Healthcare, Buckinghamshire, UK).

After visualisation, the immunoblots were routinely washed in 40% (v/v) methanol for 30 minutes to remove the precipitated fluorophore and rinsed three times, for 10 minutes, in TBS-T. The antibodies were then stripped off by incubating the PVDF membrane in 20 ml stripping buffer (25 mM Tris (pH 7.0), 8% (w/v) SDS, 0.72 M β -mercaptoethanol) at 80 °C for 30 minutes. After a further three 10 minute rinses in TBS-T, the PVDF membranes were either blocked prior to being probed with another antibody or dried and kept until such times as they were needed. Representative immunoblots are presented throughout this thesis.

2.19 Measurement of Intracellular cAMP Accumulation

Untransfected $G\alpha_{q/11}$ knockout MEF cells were seeded onto 12 well cell culture dishes (1 ml/well) at a density of 3×10^5 cells/ml and allowed to attach overnight. The growth medium was removed and the cells were washed twice in PBS prior to being incubated in serum free medium for 16 hours. GnRH stimulations were performed at 37 °C in fresh serum free medium after a 30 minute incubation with 1 mM 3-isobutyl-1-methylxanthine (IBMX). Following appropriate GnRH stimulation, the cell monolayers were placed on ice, washed twice in cold PBS and lysed in 0.1 M HCl. Intracellular cAMP concentrations were determined using an enzyme linked immunoassay kit (Biomol, Exeter, UK) as per the manufacturers instructions.

2.20 Measurement of Cell Growth

The growth medium was removed from appropriately transfected $G\alpha_{q/11}$ knockout MEF cells and the cells were incubated in fresh growth medium for 16 hours in the presence or absence of chemical inhibitors as described in the figure legends. GnRH stimulations were performed at 37 °C in fresh growth medium in the presence or absence of chemical inhibitors as described in the figure legends. Following appropriate GnRH stimulation, the cell monolayers were placed on ice and an equal volume cold 25% (v/v) trichloroacetic acid was added directly to the growth medium. The cells were fixed at 4 °C for at least 1 hour after which cell growth was determined using the SRB assay as previously described (Skehan et al., 1990, Morgan et al., 2008, White et al., 2008a). Briefly, the fixed cells were stained with 0.4% (w/v) SRB in 1% (v/v) acetic acid for at least 30 minutes prior to being washed four times in 1% (v/v) acetic acid and allowed to dry. The protein-bound dye was extracted with 10 mM Tris (pH 10.5) and the optical density determined at 540 nm using a spectrophotometer.

2.21 Trypan Blue Dye Exclusion

The growth medium was removed from appropriately transfected $G\alpha_{q/11}$ knockout MEF cells. GnRH stimulations were performed at 37 °C in fresh growth medium. Following appropriate GnRH stimulation, the cell monolayers were placed on ice and the media collected. Samples were subjected to centrifugation at 6,000 x g for 10 minutes and the supernatant was removed. The pellets were resuspended in PBS and an equal volume 0.4% (v/v) Trypan Blue solution was added. After 10 minutes, the samples were imaged on an Olympus Provis AX70 inverted light microscope (Olympus UK, Hertfordshire, UK) to test for dye uptake.

2.22 Dual Light Luciferase Reporter Gene Assay

BHK cells and L β T2 cells were transfected with Fugene as described. 6×10^5 cells/ml were seeded onto 12 well cell culture dishes (1 ml/well), allowed to attach overnight and transfected with 2 μ g/well plasmid DNA and 33 ng/well Renilla luciferase (Promega UK, Southampton, UK) to control for transfection efficiency. 24 hours after transfection, the growth medium was removed and the cells were

washed twice in PBS prior to being incubated in serum free medium for 16 hours in the presence or absence of chemical inhibitors as described in the figure legends. GnRH stimulations were performed at 37 °C in fresh serum free medium in the presence or absence of chemical inhibitors as described in the figure legends. Following appropriate GnRH stimulation, the cell monolayers were placed on ice, washed once in cold PBS and lysed in a passive lysis buffer (Promega UK, Southampton, UK) with constant shaking. Luciferase activity was determined using a dual light luciferase assay kit (Promega UK, Southampton, UK) and a FLUOstar OPTIMA luminometer (BMG Lab Technologies, Aylesbury, UK). The luciferase response was expressed in arbitrary units relative to the activity observed in vehicle treated control cells and normalised for Renilla luciferase activity. It was then expressed as a fold over control response as indicated in the figure legends.

2.23 Measurement of Inositol Phosphate Generation

BHK cells were transfected with Fugene as described. 6×10^5 cells/ml were seeded onto 12 well cell culture dishes (1 ml/well), allowed to attach overnight and transfected with 2 µg/well plasmid DNA. 24 hours after transfection, the growth medium was removed and the cells were washed twice in PBS prior to being incubated in inositol free DMEM (Invitrogen, Paisley, UK) supplemented with 2 mM glutamine, 50 IU/ml penicillin, 50 IU/ml streptomycin, 1% (v/v) dialysed foetal calf serum and 1 µCi/ml myo-D-[3 H]inositol (GE Healthcare, Buckinghamshire, UK) for 16 hours. Thereafter, the cells were incubated in 0.5 ml buffer A (140 mM NaCl, 20 mM HEPES, 8 mM glucose, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mg/ml BSA) containing 10 mM LiCl at 37 °C for 30 minutes. GnRH stimulations were performed at 37 °C in fresh buffer A containing 10 mM LiCl for a further 30 minutes. Following appropriate GnRH stimulation, the buffer A was removed and the reaction terminated by addition of 10 mM formic acid at 4 °C for at least 30 minutes. The [3 H]inositol phosphates were purified from the formic acid extracts by ion exchange chromatography using Dowex AG1-X8 ion exchange resin (Bio-Rad Laboratories, Hertfordshire, UK). The bound [3 H]inositol phosphates were eluted with 1 M ammonium formate containing 0.1 M formic acid and quantified by liquid scintillation spectroscopy.

2.24 Immunocytochemistry and Confocal Laser Microscopy

Gα_{q/11} knockout MEF cells were seeded onto 8 well chamber slides (0.5 ml/well) at a density of 0.5×10^5 cells/ml and allowed to attach overnight. The growth medium was removed and the cells were washed twice in PBS prior to being incubated in serum free medium for 16 hours in the presence or absence of chemical inhibitors as described in the figure legends. GnRH stimulations were performed at 37 °C in fresh serum free medium in the presence or absence of chemical inhibitors as described in the figure legends. Following appropriate GnRH stimulation, the cell monolayers were placed on ice, washed once in cold PBS and fixed with cold methanol at -20 °C for 10 minutes. The fixed cells were washed twice in PBS, permeabilised with immunocytochemistry permeabilisation buffer (10% (v/v) foetal calf serum, 1% (w/v) BSA, 0.2% (v/v) Nonidet P-40, PBS) for 30 minutes, washed a further two times in PBS and blocked with immunocytochemistry blocking buffer (10% (v/v) foetal calf serum, 1% (w/v) BSA, PBS) for 1 hour. The immunocytochemistry blocking buffer was discarded and a primary antibody (diluted 1:100 in immunocytochemistry blocking buffer) applied at 4 °C overnight. Each primary antibody was freshly prepared for each experiment. After appropriate incubation, the cells were washed three times in PBS prior to the application of an appropriate FITC conjugated secondary antibody (diluted 1:200 in immunocytochemistry blocking buffer) for 1 hour. After three further PBS washes, the cells were exposed to DAPI (diluted 1:2,000 in PBS) for 5 minutes in order to stain DNA, washed another three times in PBS, mounted in Permafluor mounting medium (Immunotech, Marseille, France) and left to dry in the dark for at least 48 hours. Confocal microscopy was performed on a Zeiss LSM 510 laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany) using a 40 x 1.4 numerical aperture oil immersion lens. Images were exported to Adobe Photoshop version 9.0.2 (Adobe Systems Europe, Uxbridge, UK). Representative images are presented throughout this thesis.

2.25 Statistical Analysis

All experiments were repeated independently at least three times. In addition, all assays were performed in triplicate. Data are presented as mean values + or ± the standard error of the mean (SE). Statistical significance was set at $p < 0.05$, $p < 0.01$ or

$p < 0.001$, indicated by asterisks in figures, and analyses were performed using the students t-test. For agonist dose-response analyses, data representing the mean \pm SE from at least three independent experiments were plotted and analysed using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). Sigmoidal dose response curves were fitted to the relevant data sets and the EC₅₀ value determined.

Chapter Three

A Crucial Role for $G\alpha_{q/11}$, but not $G\alpha_{i/o}$ or $G\alpha_s$, in GnRH Receptor-Mediated Cell Growth Inhibition

3.0 Abstract

GnRH acts on its cognate receptor in pituitary gonadotropes to regulate the biosynthesis and the secretion of gonadotropins. However, it may also inhibit cellular growth in reproductive malignancies and, here, the GnRH-mediated activation of the MAPK cascades is thought to play a pivotal role. In extrapituitary tissues, GnRH receptor signalling has been postulated to involve the coupling of the receptor to different G proteins. In this chapter, I examined the ability of the GnRH receptor to couple directly to $G_{q/11}$, $G_{i/o}$ and G_s , their roles in the activation of the MAPK cascades and the subsequent effects on cellular growth. I show that, in $G_{q/11}$ knockout cells stably expressing the GnRH receptor, GnRH did not induce detectable activation of ERK, JNK or P38. In contrast to G_{α_i} or chimeric $G_{\alpha_{q15}}$, transfection of G_{α_q} cDNA enabled GnRH to induce phosphorylation of all of these proteins. Furthermore, no GnRH-mediated cAMP response or inhibition of isoproterenol-induced cAMP accumulation was observed. Interestingly, GnRH I stimulated a marked reduction in cell growth only in cells expressing G_{α_q} . This inhibition could be significantly rescued by blocking ERK activation with PD98059 whilst the inhibition of PKC with Ro-31-8220, the chelation of intracellular Ca^{2+} with BAPTA-AM, the inhibition of JNK with SP600125 or the inhibition of P38 with SB203580 had no effect. Notably, specific inhibition of src Tyr kinase with PP2 was sufficient to completely abrogate the GnRH I-induced antiproliferative response. I therefore provide evidence to suggest that the coupling of the GnRH receptor to $G_{q/11}$, but not to $G_{i/o}$ or G_s , and the consequent activation of the src and the ERK pathways (but neither the JNK nor the P38 cascades) plays a crucial role in GnRH I-mediated antiproliferation. Based on these and other data, I propose a mechanism whereby GnRH I may inhibit cellular growth.

3.1 Introduction

As well as influencing reproductive behaviour, evidence suggests that GnRH may act peripherally to exert a growth regulatory effect on certain cell types. Indeed, GnRH and the GnRH receptor have been found in extrapituitary tissues such as the ovary (Oikawa et al., 1990, Fraser et al., 1996) and the mammary gland (Palmon et al., 1994). Cancers of the breast (Baumann et al., 1993, Kakar et al., 1994, Mangia et al.,

2002), ovary (Kakar et al., 1994, Irmer et al., 1995), endometrium (Borri et al., 1998) and prostate (Limonta et al., 1999) have also been shown to express both the ligands and the receptor. Several studies, both *in vitro* and *in vivo*, have demonstrated that the direct application of GnRH analogues to receptor expressing cells results in an attenuation of cellular proliferation and the activation of proapoptotic signalling mechanisms (Klijn and de Jong, 1982, Dondi et al., 1994, Yano et al., 1992, Yano et al., 1994, Jeyarajah et al., 1996, Dondi et al., 1998, Kimura et al., 1999, Kraus et al., 2004, Maudsley et al., 2004, Gnanapragasam et al., 2005, Morgan et al., 2008, White et al., 2008a, White et al., 2008b). In such GnRH-induced cellular responses, activation of the MAPK cascades is sometimes thought to play a fundamental role (Kimura et al., 1999, Kraus et al., 2004, Maudsley et al., 2004, Dobkin-Bekman et al., 2006, White et al., 2008a).

Several groups have demonstrated that GnRH stimulates the phosphorylation of ERK, JNK and P38 in the α T31 and the L β T2 gonadotrope cell lines and a wide variety of GnRH receptor transfected cells (Sim et al., 1993, Sim et al., 1995, Reiss et al., 1997, Haisenleder et al., 1998, Levi et al., 1998, Roberson et al., 1999, Naor et al., 2000, Harris et al., 2002, Maudsley et al., 2004, Dobkin-Bekman et al., 2006, White et al., 2008a). How these cascades are initiated upstream by the activated receptor and which of them impinge on cell growth inhibition remains unclear. It has been proposed that, whereas the actions of GnRH at the pituitary are mediated by the interaction of the receptor with the G protein subunits of the $G_{q/11}$ family and the consequent signalling to and the activation of, among other molecules, ERK, JNK and P38, the antiproliferative actions of GnRH are best explained via an interaction of the receptor with the $G_{\alpha_{i/o}}$ G protein α subunits. Indeed, Imai and colleagues demonstrated that treatment with PTX of plasma membrane preparations from surgically removed ovarian carcinomas and uterine leiomyosarcomas completely inhibits the buserelin-mediated stimulation of the phosphotyrosine phosphatases (PTPs) (Imai et al., 1996). The authors further speculated that, as PTP activity has been proposed to be involved in the antiproliferative action of dopamine at the D_2 receptor (Florio et al., 1992), this observation may have relevance to the GnRH-induced inhibition of cell growth. Additionally, Limonta and colleagues showed that

PTX completely abrogates the inhibitory effects of GnRH analogues (zoladex) on cellular proliferation in LNCaP and DU145 cells (Limonta et al., 1999). With regards to the involvement of the MAPK cascades in the GnRH-mediated induction of cell growth inhibition, Kraus and colleagues implicated the activation of JNK in the GnRH analogue- ([D-Trp⁶]-GnRH) induced antiproliferative effect (Kraus et al., 2004). Inhibition of JNK activation in DU145 cells with SP600125 completely abrogated the [D-Trp⁶]-GnRH elicited terminal deoxynucleotidyltransferase-mediated nick end labelling (TUNEL) response. Similarly, Maudsley and colleagues showed that, depending on the cell line studied, GnRH-induced inhibition of cell growth coincided with the activation of either JNK (JEG3) or P38 (BPH1) (Maudsley et al., 2004). Inhibition of the activation of these kinases significantly reduced the GnRH I-mediated annexin 5-FITC staining of phosphatidylserine expressed on the outer aspect of the plasma membrane. Interestingly, these results are in contrast to those obtained by Kimura and colleagues who showed that, in the Caov3 cell line, specific inhibition of the ERK cascade with PD98059 was sufficient to completely impede leuprolide-induced antiproliferation (Kimura et al., 1999). Viewed collectively, these data suggest that interaction of the GnRH receptor with different G proteins may explain the published diversity of the mechanisms involved in MAPK activation and the subsequent effects on cellular fate. However, as reviewed in chapter one, this theory is not without opposition and, despite largely circumstantial evidence, convincing proof of the activation of multiple G proteins by the agonist-bound GnRH receptor is still severely lacking.

To better understand the pathways involved in GnRH-mediated cell growth inhibition, I set out to clarify the G protein coupling profile of the GnRH receptor. I provide evidence that the receptor couples to G proteins of the G_{q/11} family but not to G_{i/o} or G_s as previously suggested. I also demonstrate that ERK, JNK and P38 activation, in response to GnRH treatment, may be mediated by G_{q/11} and that G_{q/11} may facilitate the induction of antiproliferative signalling by GnRH I. Moreover, I show that the src and the ERK cascades, but neither the JNK nor the P38 pathways, also play a pivotal role in this process.

3.2 Results

3.2.1 GnRH I-Induced MAPK Activation may be Mediated by $G_{q/11}$

In order to facilitate the potential coupling of the GnRH receptor to G proteins other than $G_{q/11}$, I performed a series of experiments in $G_{\alpha_{q/11}}$ knockout MEF cells stably expressing the receptor protein. These cells thus eliminate the possible competition from $G_{q/11}$ for the binding of this GPCR. The $G_{\alpha_{q/11}}$ knockout MEF cell line expresses more receptors per cell than either the SCL60 cells or the L β T2 cells whilst the binding affinity of GnRH I is not significantly different (figure 3.1; table 3.1). Interestingly, the estimated number of GnRH binding sites on α T31 cells is approximately 50% of the number on primary gonadotropes (Kaiser et al., 1997). Thus, the $G_{\alpha_{q/11}}$ knockout MEF cell model may better reflect the GnRH receptor expression levels encountered *in vivo*.

To elucidate the ability of GnRH to activate ERK, JNK and P38 in these cells, GnRH I was applied in both time- and dose-dependent manners. Stimulation brought about no significant increase in the levels of pERK1/2, pJNK1 or pP38 at any time or dose tested. Transient expression of G_{α_q} allowed GnRH I to elicit an increase in ERK1/2 phosphorylation with a maximal response of 3.0 ± 0.4 -fold that of vehicle treated controls after 5 minutes stimulation (figure 3.2). Additionally, JNK1 and P38 phosphorylation became evident giving maximal responses of 3.7 ± 0.3 -fold after 5 minutes stimulation (figure 3.3) and 3.6 ± 0.2 -fold after 30 minutes stimulation (figure 3.4) respectively. Agonist dose-response analysis yielded EC_{50} values for the induction of pERK1/2, pJNK1 and pP38, after 10 minutes stimulation, of 1.0 nM (figure 3.5), 3.3 nM (figure 3.6) and 2.4 nM (figure 3.7) GnRH I respectively.

In vector transfected cells, the complete absence of $G_{\alpha_{q/11}}$ was verified both by western blotting (figure 3.8) and the lack of phosphoinositide (PhI) hydrolysis (figure 3.9). When compared to L β T2 cells, the levels of G_{α_q} following transient G protein expression were within the physiological range. Additionally, transient transfection of G_{α_q} allowed GnRH I to elicit an E_{max} PhI response of 17.6 ± 0.5 -fold that of vehicle treated controls. In contrast, the E_{max} PhI response obtained in cells transfected with vector was 1.4 ± 0.5 -fold.

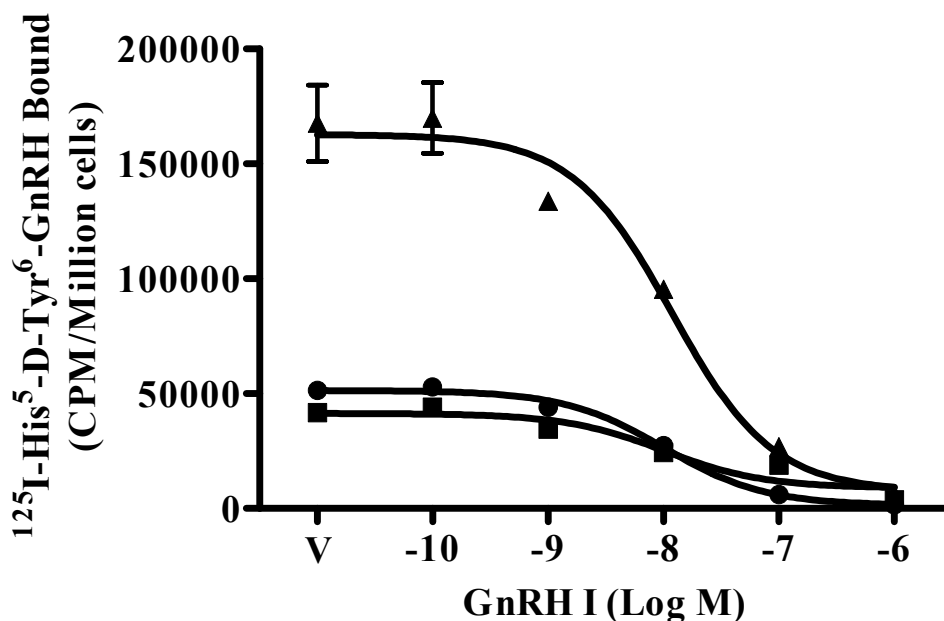


Figure 3.1 Radioligand binding assays depicting the expression levels of the GnRH receptor. Intact $G_{\alpha q/11}$ knockout MEF cells, stably expressing the GnRH receptor, (▲), SCL60 cells (●) and L β T2 cells (■) were incubated with ^{125}I -[His⁵,D-Tyr⁶]GnRH (100,000 cpm/0.5 ml/well) and either vehicle (0.2% propylene glycol; V) or increasing doses of unlabelled GnRH I (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μM) as indicated for 4 hours at 4°C. Data are representative of at least three independent experiments and the mean counts/minute/million cells (CPM/Million cells) \pm SE is presented.

Cell Line	IC ₅₀ (nM)
$G_{\alpha q/11}$ knockout MEF	11.1 \pm 2.2
SCL60	10.1 \pm 1.7
L β T2	10.1 \pm 1.3

Table 3.1 GnRH I binding affinities. Data from at least three independent experiments were quantified and the mean IC₅₀ \pm SE is presented.

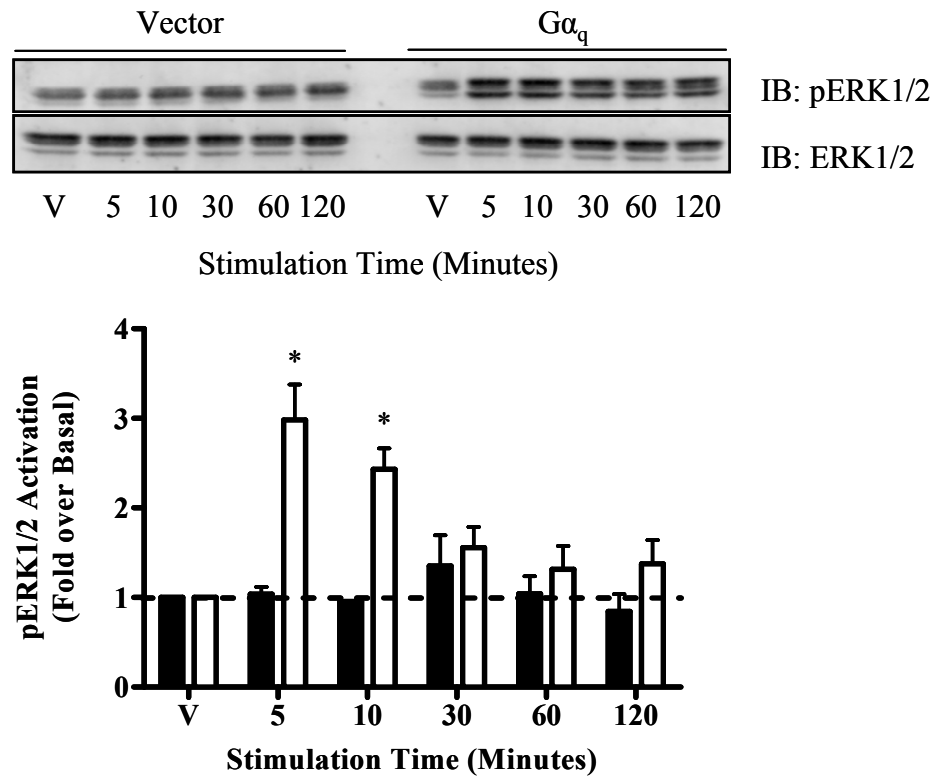


Figure 3.2 Immunoblots depicting the time dependence of the GnRH I-induced phosphorylation of ERK1/2. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (black bars) or $G\alpha_q$ (white bars) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I for the indicated times. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of ERK1/2 is presented. $p < 0.05$ (*) represents statistical significance from vehicle treated controls.

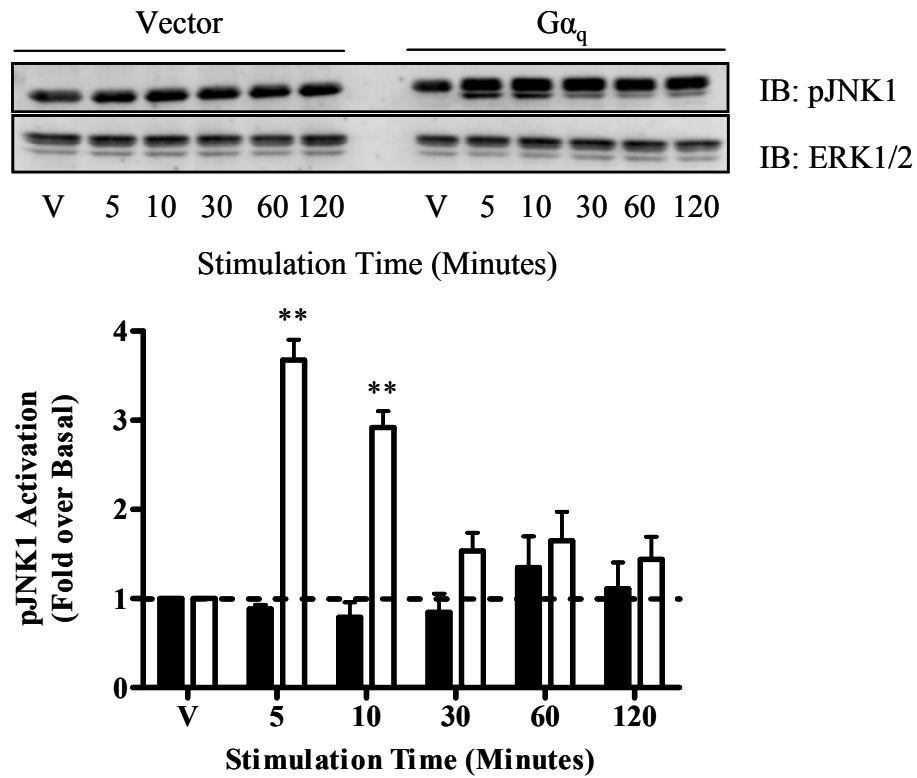


Figure 3.3 Immunoblots depicting the time dependence of the GnRH I-induced phosphorylation of JNK1. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (black bars) or $G\alpha_q$ (white bars) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I for the indicated times. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of JNK1 is presented. $p < 0.01$ (**) represents statistical significance from vehicle treated controls.

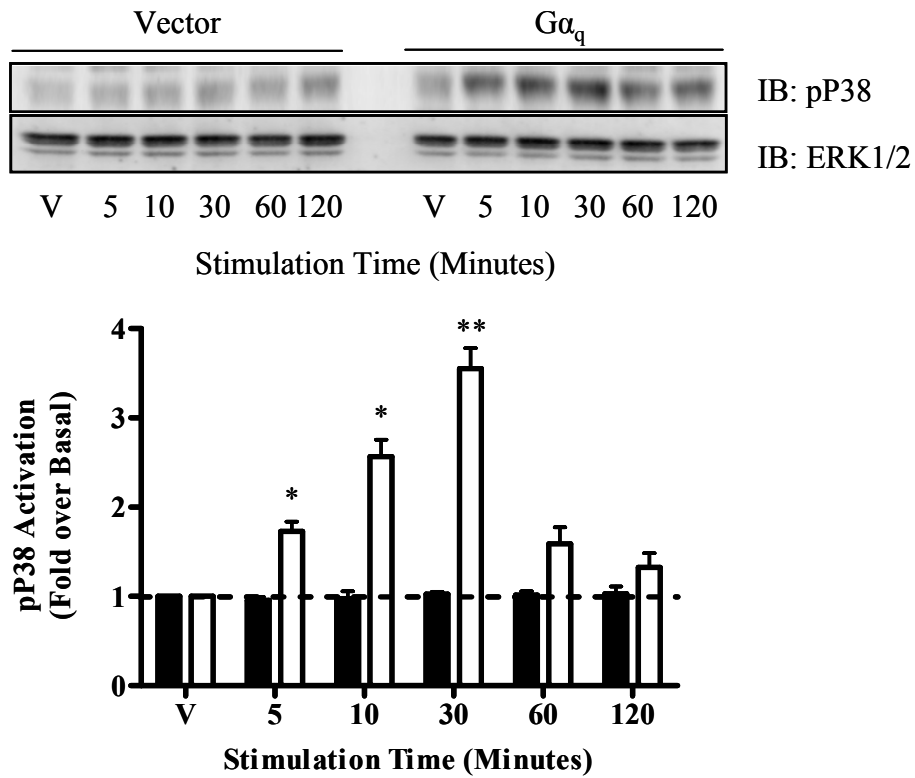


Figure 3.4 Immunoblots depicting the time dependence of the GnRH I-induced phosphorylation of P38. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (black bars) or $G\alpha_q$ (white bars) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I for the indicated times. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of P38 is presented. $p < 0.05$ (*) and $p < 0.01$ (**) represents statistical significance from vehicle treated controls.

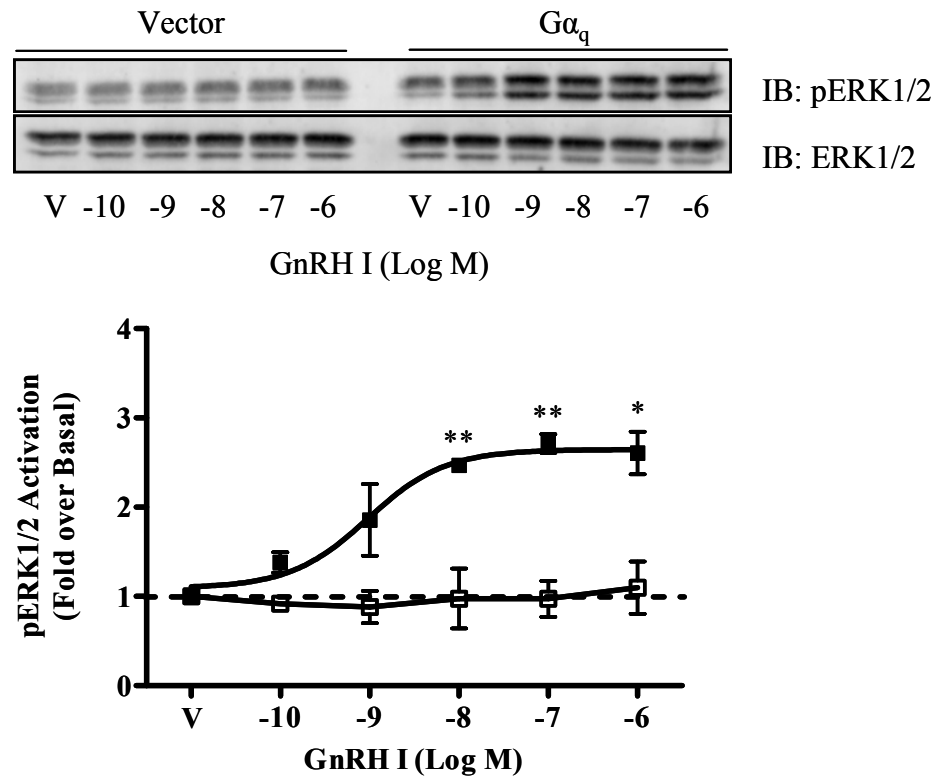


Figure 3.5 Immunoblots depicting the dose dependence of the GnRH I-induced phosphorylation of ERK1/2. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (□) or $G\alpha_q$ (■) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or increasing doses of GnRH I (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μ M) as indicated for 10 minutes. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of ERK1/2 is presented. $p < 0.05$ (*) and $p < 0.01$ (**) represents statistical significance from vehicle treated controls.

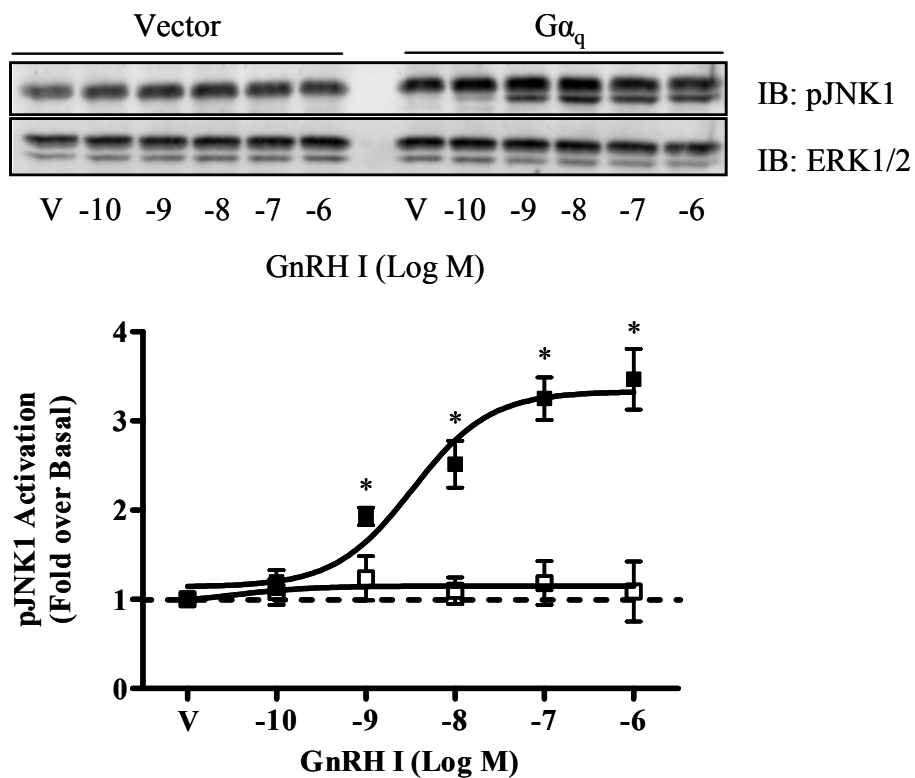


Figure 3.6 Immunoblots depicting the dose dependence of the GnRH I-induced phosphorylation of JNK1. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (□) or $G\alpha_q$ (■) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or increasing doses of GnRH I (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μ M) as indicated for 10 minutes. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of JNK1 is presented. $p < 0.05$ (*) represents statistical significance from vehicle treated controls.

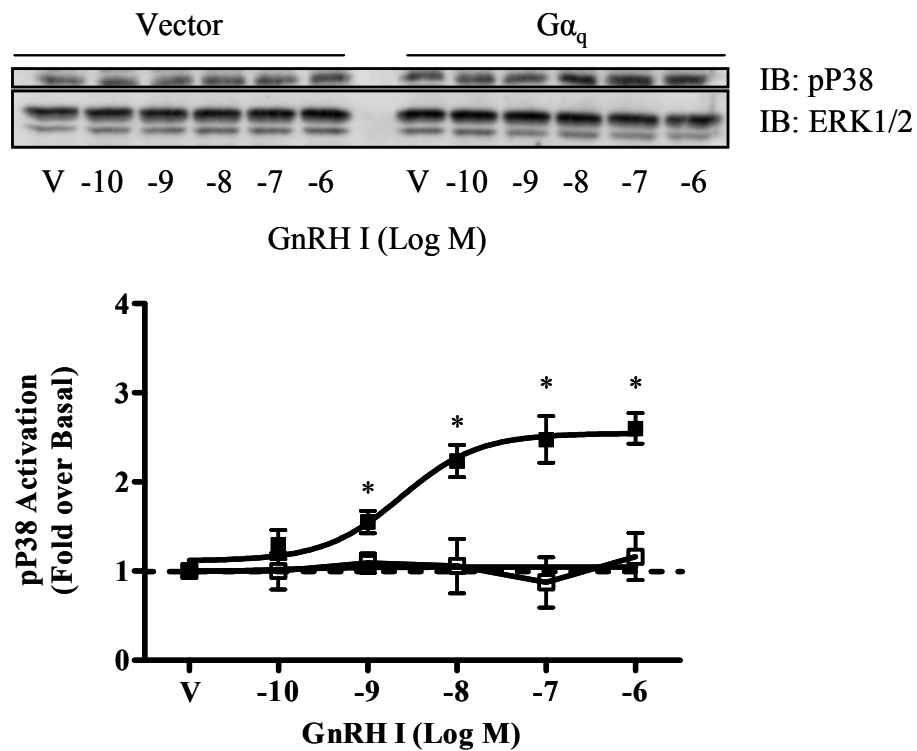


Figure 3.7 Immunoblots depicting the dose dependence of the GnRH I-induced phosphorylation of P38. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (□) or $G\alpha_q$ (■) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or increasing doses of GnRH I (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μ M) as indicated for 10 minutes. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of P38 is presented. $p < 0.05$ (*) represents statistical significance from vehicle treated controls.

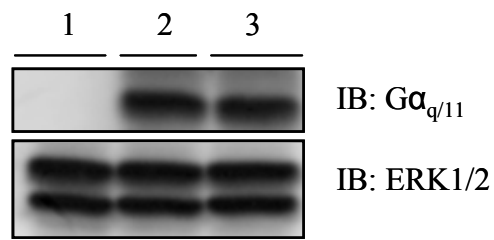


Figure 3.8 Immunoblots depicting the relative $G\alpha_q$ expression levels. $G\alpha_{q/11}$ knockout MEF cells transiently transfected with vector (lane 1) or $G\alpha_q$ (lane 2) cDNA and L β T2 cells (lane 3) were serum starved for 16 hours. Unstimulated cell lysates were collected. Representative blots are shown.

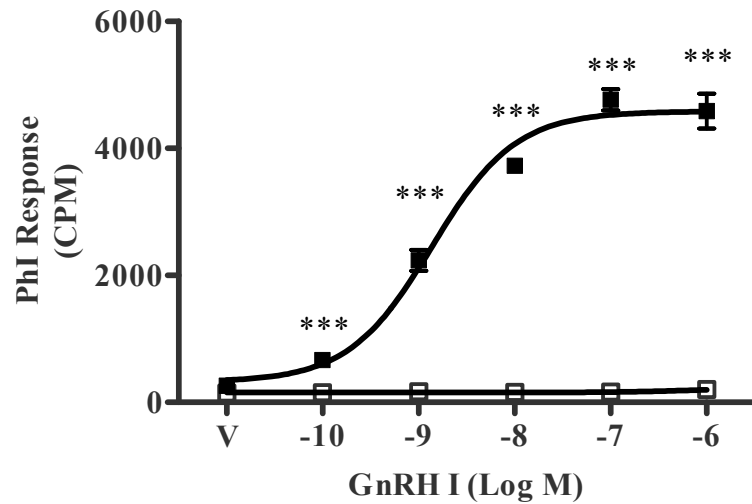


Figure 3.9 Phosphoinositide hydrolysis assays depicting the functionality of transfected $G\alpha_q$. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (\square) or $G\alpha_q$ (\blacksquare) cDNA were labelled overnight with 1 μ Ci/ml myo-D-[3 H]inositol prior to being treated with vehicle (0.2% propylene glycol; V) or increasing doses of GnRH I (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μ M) as indicated for 30 minutes. Data are representative of at least three independent experiments and the mean counts/minute (CPM) \pm SE for phosphoinositide hydrolysis is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls.

3.2.2 The GnRH Receptor does not Directly Interact with $G_{i/o}$ or G_s

Thus far I have confirmed that the transfection of $G\alpha_q$ into the $G\alpha_{q/11}$ knockout MEF cell model allows the detection of significant GnRH-induced activation of ERK, JNK and P38. Nevertheless, previous studies have argued that these responses may also be mediated by the interaction of the GnRH receptor with other G protein α subunits (Sim et al., 1995, Kimura et al., 1999, Maudsley et al., 2004). Although my previous results indicate that (in the complete absence of $G\alpha_{q/11}$) it was not possible to detect significant increases in MAPK activation, I attempted to address this theory. Initially, I considered the possibility that the overexpression of $G\alpha_i$ may facilitate detectable MAPK signalling. In contrast to $G\alpha_q$, transient transfection of $G\alpha_{i1}$, $G\alpha_{i2}$ or $G\alpha_{i3}$ did not enable GnRH I to significantly increase ERK1/2, JNK1 or P38 phosphorylation (figure 3.10). Additionally, I made use of a chimeric G protein α subunit; $G\alpha_{qi5}$. The carboxy-terminus of the G protein α subunit has previously been shown to play a pivotal role in defining the specificity of receptor-G protein interaction (Conklin et al., 1993). Mutation of the five carboxy-terminal amino acids is sufficient to completely switch GPCR coupling selectivity. The cDNA of $G\alpha_{qi5}$ codes for a G protein α subunit consisting of mainly $G\alpha_q$. The five carboxy-terminal amino acids have, however, been substituted for those from $G\alpha_{i2}$. This facilitates $G_{i/o}$ -coupled GPCR signalling through PLC β and, as such, a classical $G\alpha_q$ -mediated output is indicative of receptor- $G_{i/o}$ interaction. Transient expression of $G\alpha_{qi5}$ in the $G\alpha_{q/11}$ knockout MEF cell line did not enable the activated receptor to elicit any increase in ERK1/2, JNK1 or P38 phosphorylation (pERK1/2 - 1.0 ± 0.1 -fold; pJNK1 - 1.1 ± 0.2 -fold; pP38 - 1.0 ± 0.1 -fold).

In order to address the possibility that the GnRH receptor does interact with $G_{i/o}$, but that $G_{i/o}$ does not activate the MAPK cascades thus making it impossible to identify such interaction using MAPK phosphorylation as an output, I assessed the ability of GnRH to inhibit intracellular cAMP accumulation. Stimulation of the $G\alpha_{q/11}$ knockout MEF cells with isoproterenol increased intracellular cAMP levels to 14.7 ± 0.4 -fold that of vehicle treated controls (figure 3.11). Stimulation with isoproterenol and GnRH I together did not significantly affect the cAMP response obtained ($15.5 \pm$

0.7-fold). Notably, stimulation with GnRH I alone was also without effect (0.8 ± 0.2 -fold).

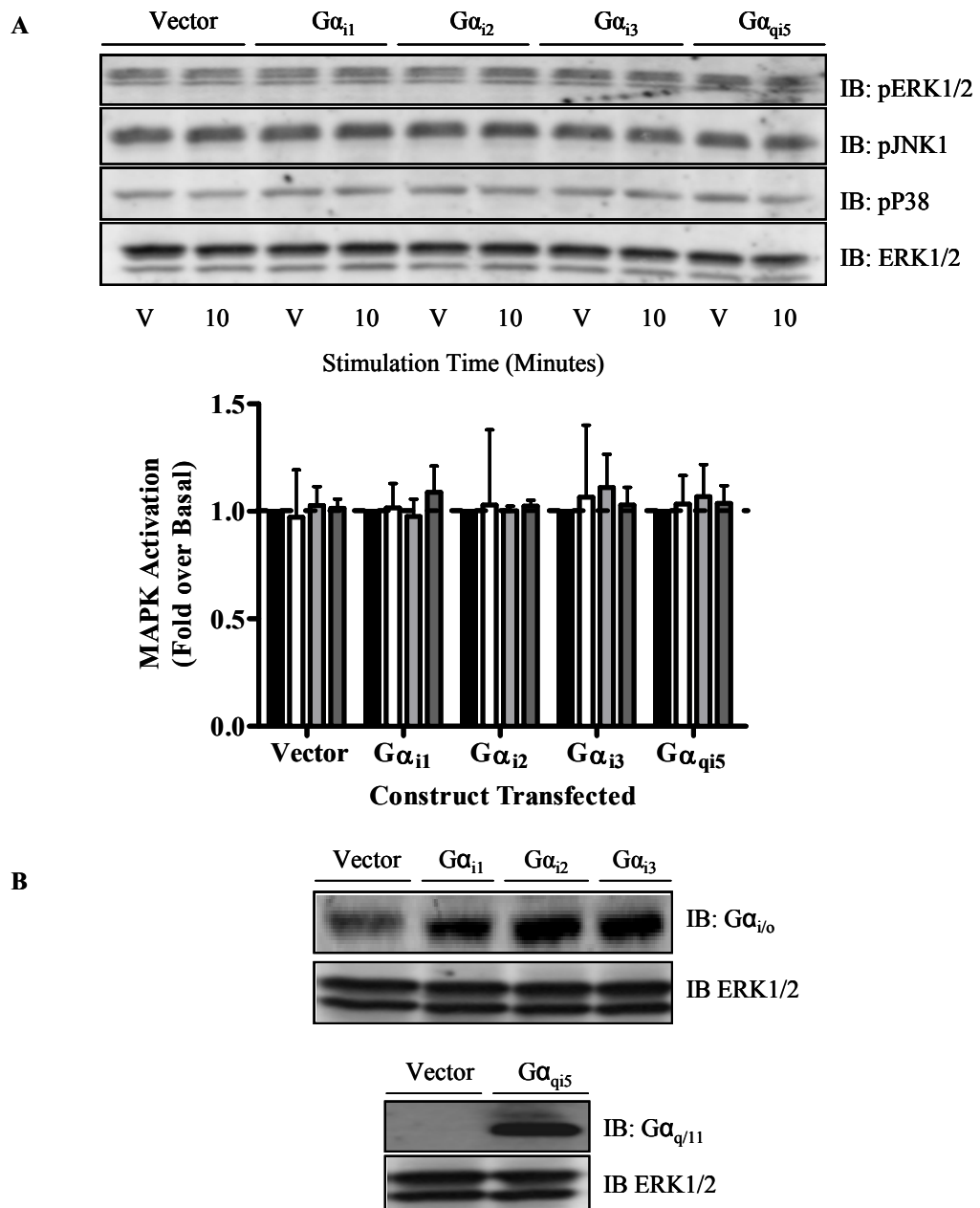


Figure 3.10 Immunoblots depicting the lack of effect of transient overexpression of $G\alpha_{i1-3}$ or $G\alpha_{qi5}$ on the GnRH I-induced phosphorylation of ERK1/2, JNK1 and P38. A; $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_{qi5}$ cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V; black bars) or 1 μ M GnRH I as indicated for 10 minutes. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of ERK1/2 (white bars), JNK1 (light grey bars) and P38 (dark grey bars) is presented. B; $G\alpha_{q/11}$ knockout MEF cells transiently transfected with vector, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_{qi5}$ cDNA were serum starved for 16 hours. Unstimulated cell lysates were collected. Representative blots are shown.

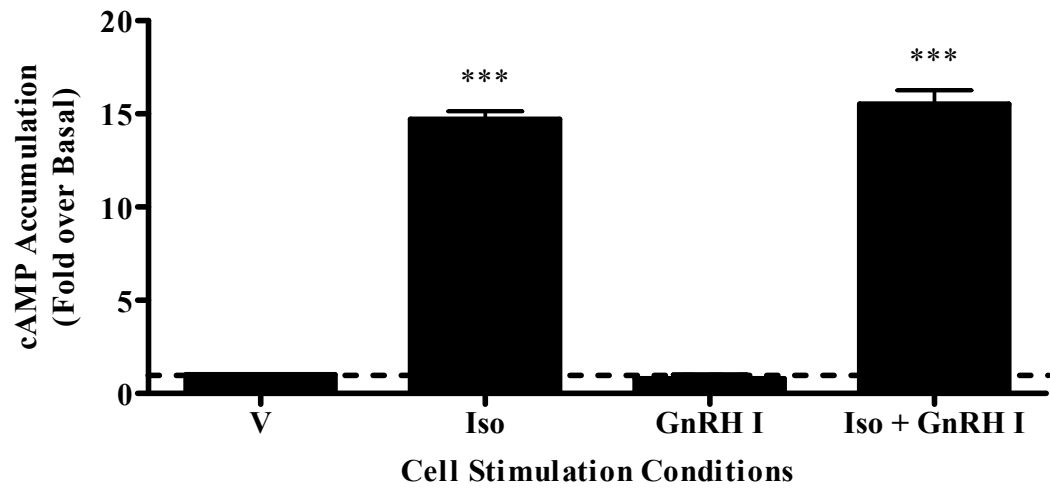


Figure 3.11 cAMP assays depicting the effect of GnRH I and isoproterenol on intracellular cAMP accumulation. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V), 3 μ M isoproterenol (Iso), 1 μ M GnRH I or 1 μ M GnRH I and 3 μ M isoproterenol as indicated for 30 minutes after a 30 minute incubation with 1 mM IBMX. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the stimulation of intracellular cAMP accumulation is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls.

3.2.3 Coupling of the GnRH Receptor to $G_{q/11}$ and the Activation of ERK is Involved in GnRH I-Mediated Cell Growth Inhibition

Continuous treatment of the $G_{q/11}$ knockout MEF cells with GnRH I resulted in a time- and dose-dependent inhibition of cell growth (relative to that of vehicle treated controls) only in cells transfected with G_{α_q} (figure 3.12). In these cells, significant growth inhibition was evident on day 1 (cell growth was $94 \pm 1\%$) and increased in a time-dependent manner to reach a maximum inhibition on day 4 (cell growth was $70 \pm 3\%$). A P38 inhibitor, SB203580, (cell growth was $75 \pm 2\%$ on day 4) (figure 3.13) and a JNK inhibitor, SP600125, (cell growth was $64 \pm 14\%$ on day 4) (figure 3.14) were unable to significantly rescue this inhibition but a MEK1/2 inhibitor, PD98059, (cell growth was $90 \pm 2\%$ on day 4) (figure 3.15) significantly decreased the GnRH I-induced effect. Agonist dose-response analysis yielded an IC_{50} value for the induction of cell growth inhibition on day 4 in cells transfected with G_{α_q} of 4.5 nM GnRH I (figure 3.16). Vector transfected control cells showed no significant inhibition of growth in response to GnRH I treatment nor did any of the MAPK inhibitors have a significant effect. Trypan blue dye exclusion confirmed that the detached cells were dead (as measured by dye uptake when compared to either live cell suspensions or vector transfected samples) and that these dead cells were more numerous than in the samples from vector transfected controls (figure 3.17).

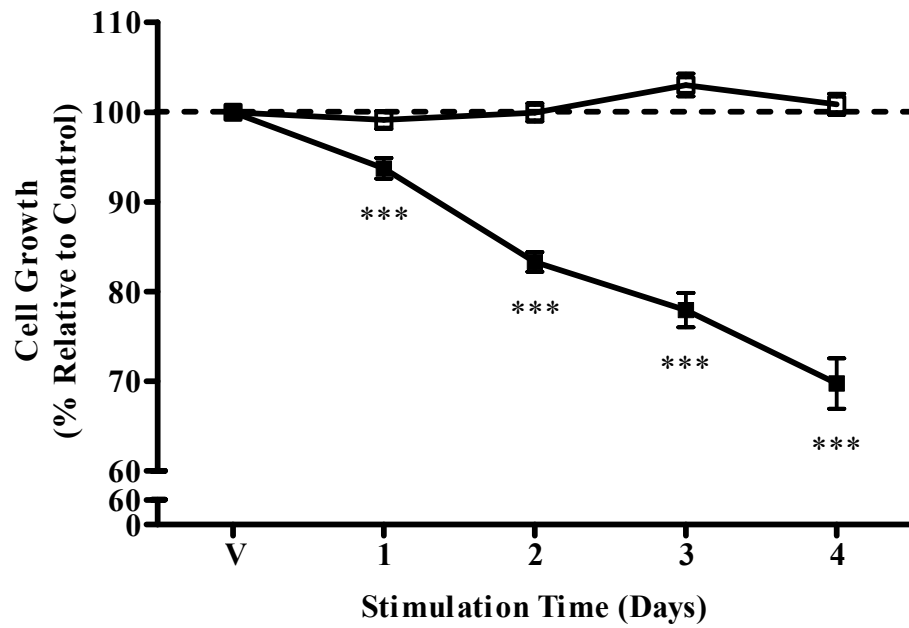


Figure 3.12: Sulforhodamine B assays depicting the time dependence of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□) or $G\alpha_q$ (■) were incubated in media containing 10% serum prior to being treated with vehicle (0.2% propylene glycol; V) or 100 nM GnRH I for the indicated times. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls.

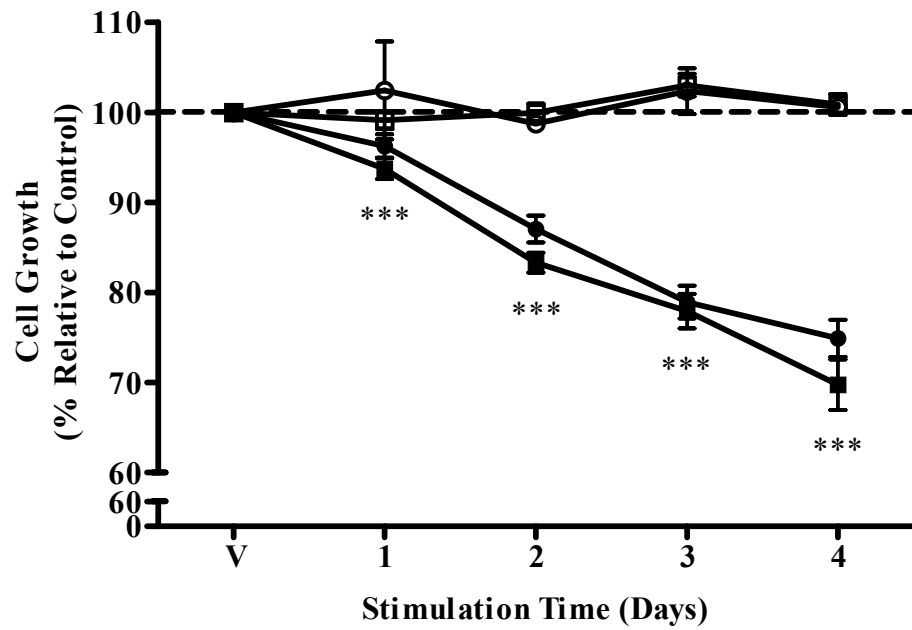


Figure 3.13: Sulforhodamine B assays depicting the SB203580 insensitivity of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□, ○) or $G\alpha_q$ (■, ●) were incubated in media containing 10% serum in the presence (○, ●) or absence (□, ■) of 20 μ M SB203580 prior to being treated with vehicle (0.2% propylene glycol; V) or 100 nM GnRH I for the indicated times. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls (for $G\alpha_q$ transfected cells).

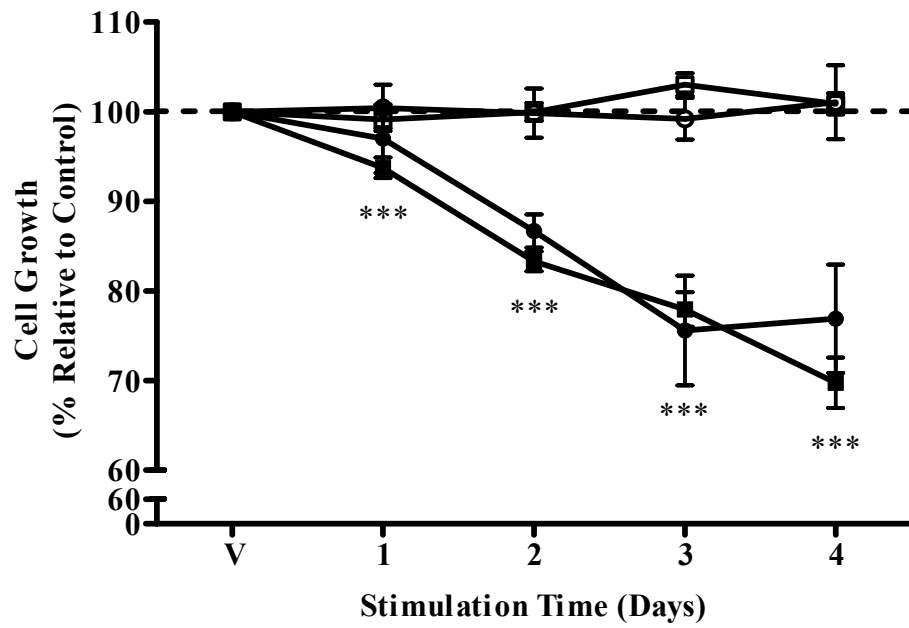


Figure 3.14: Sulforhodamine B assays depicting the SP600125 insensitivity of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□, ○) or $G\alpha_q$ (■, ●) were incubated in media containing 10% serum in the presence (○, ●) or absence (□, ■) of 50 μ M SP600125 prior to being treated with vehicle (0.2% propylene glycol; V) or 100 nM GnRH I for the indicated times. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls (for $G\alpha_q$ transfected cells).

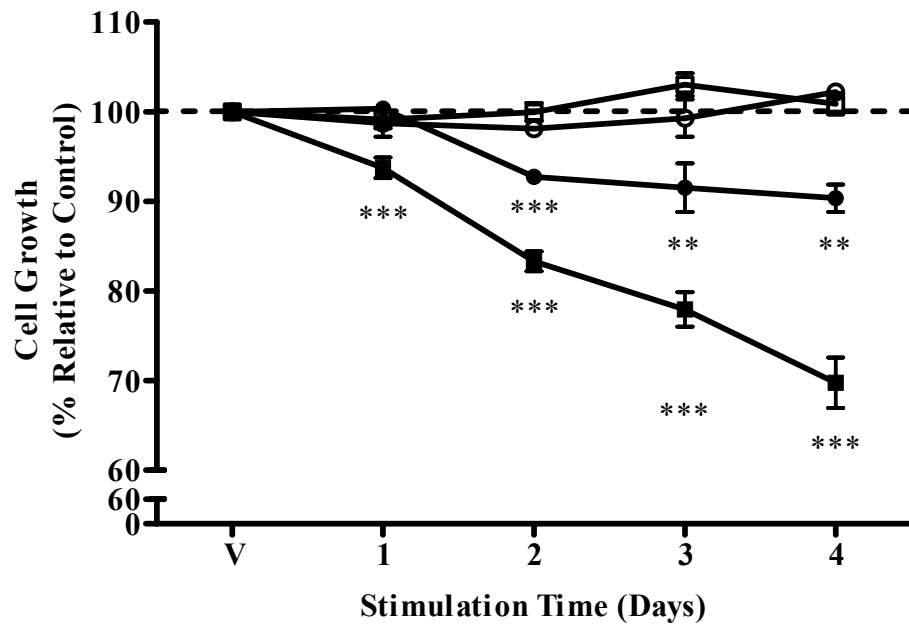


Figure 3.15: Sulforhodamine B assays depicting the PD98059 sensitivity of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□, ○) or $G\alpha_q$ (■, ●) were incubated in media containing 10% serum in the presence (○, ●) or absence (□, ■) of 50 μ M PD98059 prior to being treated with vehicle (0.2% propylene glycol; V) or 100 nM GnRH I for the indicated times. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.01$ (**) and $p < 0.001$ (***) represents statistical significance from vehicle treated controls and from GnRH I treated controls.

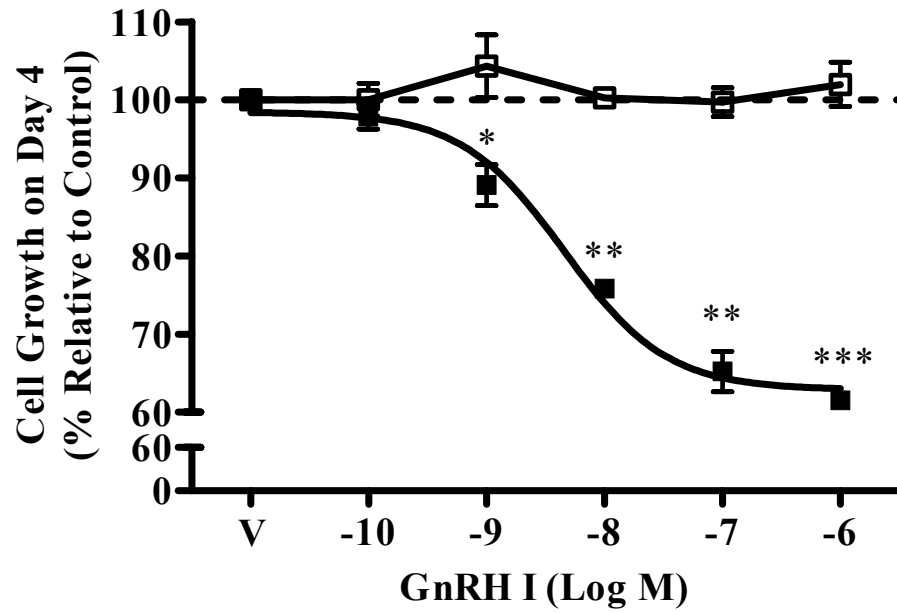


Figure 3.16: Sulforhodamine B assays depicting the dose dependence of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□) or $G\alpha_q$ (■) were incubated in media containing 10% serum prior to being treated with vehicle (0.2% propylene glycol; V) or increasing doses of GnRH I (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μ M) as indicated for 4 days. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) represents statistical significance from vehicle treated controls.

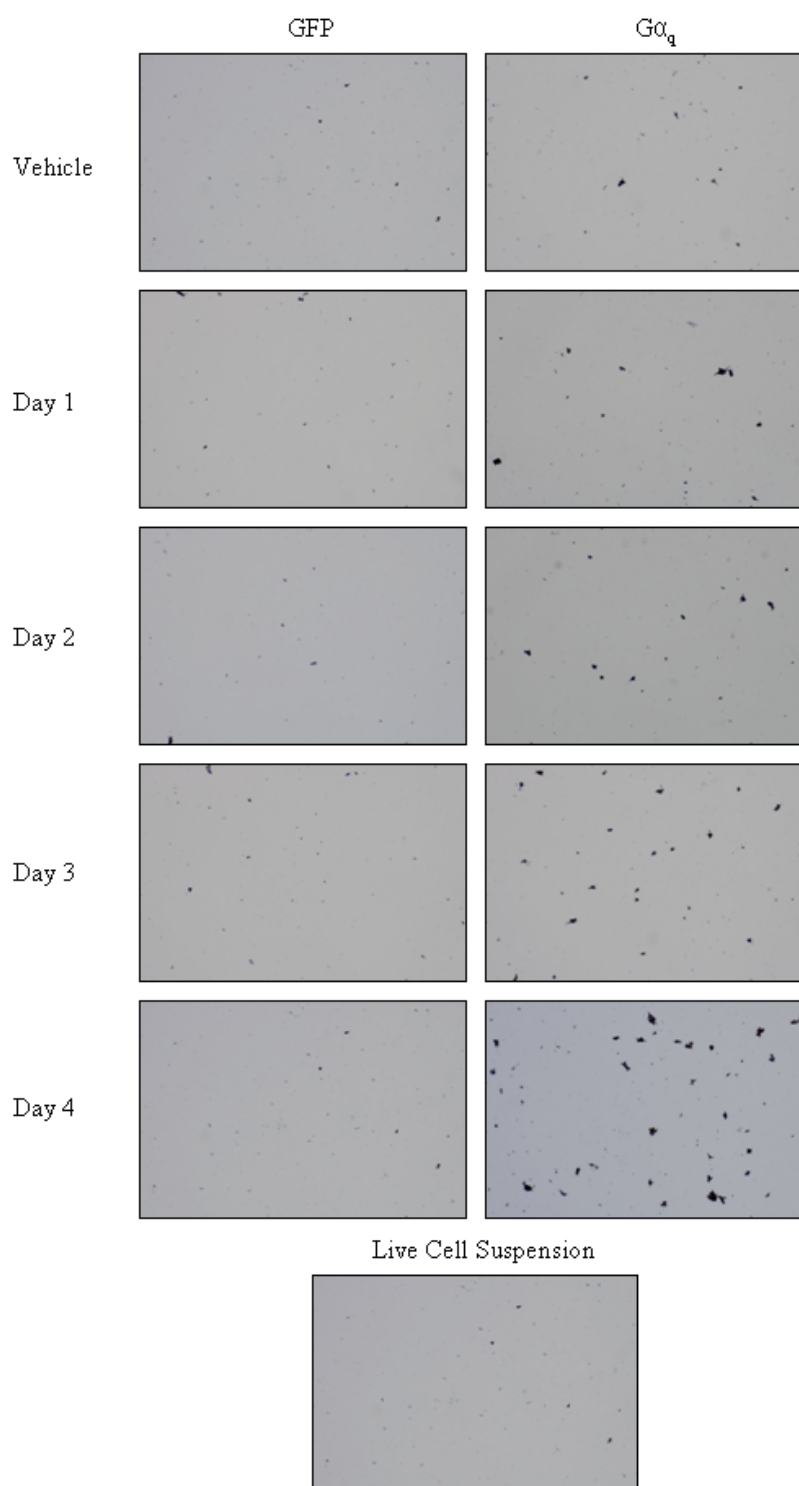


Figure 3.17 Trypan Blue dye exclusion assays depicting the time dependence of the GnRH I-induced cell death. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP or $G\alpha_q$ were incubated in media containing 10% serum prior to being treated with vehicle (0.2% propylene glycol) or 100 nM GnRH I for the indicated times. Representative images are shown.

3.2.4 GnRH I-Mediated Cell Growth Inhibition Occurs Independently of PKC and Ca^{2+} but Src Plays a Pivotal Role

Given that my previous results indicate that there exists a crucial role for $\text{G}\alpha_{q/11}$ in the GnRH I-mediated inhibition of cell growth, I attempted to further elucidate the signalling pathway involved in such a process. Activation of the $\text{G}\alpha_{q/11}$ G protein α subunits leads to the rapid and pronounced stimulation of $\text{PLC}\beta$ (Taylor et al., 1990, Lee et al., 1992a). Subsequently, PIP_2 hydrolysis to IP_3 occurs with resultant elevation of intracellular Ca^{2+} . Additionally, the same reaction results in the formation of DAG, the physiological regulator of PKC activity. PKC has been reported to have an effect on the GnRH-mediated inhibition of cell growth (Yamamoto et al., 2001, Kim et al., 2006, Morgan et al., 2008). The role of Ca^{2+} in this process, however, has not been widely investigated. In my hands, PKC inhibition with Ro-31-8220 (cell growth was $80 \pm 4\%$ on day 4) (figure 3.18) or the chelation of intracellular calcium with BAPTA-AM (cell growth was $79 \pm 2\%$ on day 4) (figure 3.19) did not in any way influence the GnRH I-induced antiproliferative response. Interestingly, a 5-fold lower concentration of BAPTA-AM has previously been shown to completely inhibit the thrombin- or bradykinin-induced increase in intracellular Ca^{2+} in the same cell type (Vogt et al., 2003) and several studies have shown significant effects of both of these inhibitors, when used at equimolar concentrations, on the GnRH-mediated signalling cascades (Mulvaney and Roberson, 2000, Maudsley et al., 2007).

These data indicate that while $\text{G}\alpha_{q/11}$ is essential for the GnRH I-induced inhibition of cell growth to occur, such inhibition does not rely on the canonical signalling pathways activated by these G protein α subunits. A recent study by Voisin and colleagues reported similar findings at the orexin OX_1 receptor (Voisin et al., 2008). The authors demonstrated that orexin-induced apoptosis is mediated by a mechanism driven by SHP-2 phosphatase recruitment to the receptor. This recruitment requires $\text{G}\alpha_{q/11}$ but occurs independently of the activity of $\text{PLC}\beta$. Moreover, the authors highlighted a critical role for src in this process. It was proposed that, while $\text{G}\alpha_{q/11}$ is necessary for the induction of the orexin-mediated proapoptotic response, src-mediated Tyr phosphorylation of the receptor protein is central to this signalling

mechanism. Upon phosphorylation, the OX_1 receptor was shown to recruit and associate with SHP-2 directly at a pYxxL motif in the intracellular end of TM7. This association was demonstrated to be critical for the orexin-mediated induction of apoptosis to occur. Given that evidence from our laboratory indicates that GnRH I may potentially activate SHP-2 in both dose- and time-dependent manners, and that src is an absolute requirement for such activation (Coetsee and Lu, unpublished observations), I sought to investigate whether src is involved in the regulation of the GnRH I-mediated inhibition of cell growth. Inhibition of src with PP2 was sufficient to completely prevent the GnRH I-induced antiproliferation in $G\alpha_{q/11}$ knockout MEF cells transiently transfected with $G\alpha_q$. In $G\alpha_q$ transfected GnRH treated controls, cell growth was $81 \pm 1\%$ after a 4 day exposure to GnRH I (figure 3.20). PP2 treatment resulted in the complete abrogation of this effect (cell growth was $101 \pm 2\%$ on day 4).

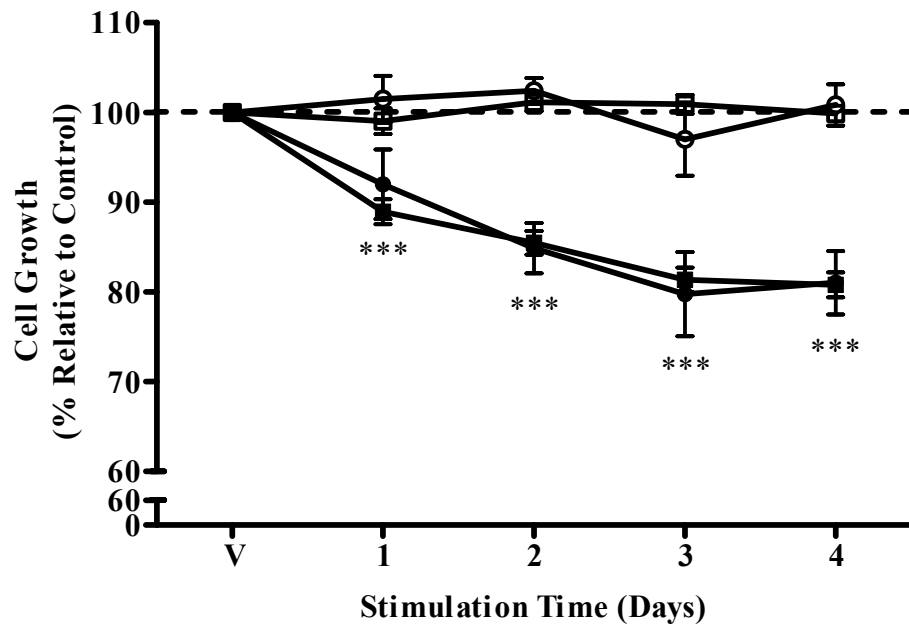


Figure 3.18: Sulforhodamine B assays depicting the Ro-31-8220 insensitivity of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□, ○) or $G\alpha_q$ (■, ●) were incubated in media containing 10% serum in the presence (○, ●) or absence (□, ■) of 100 nM Ro-31-8220 prior to being treated with vehicle (0.2% propylene glycol; V) or 100 nM GnRH I for the indicated times. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls (for $G\alpha_q$ transfected cells).

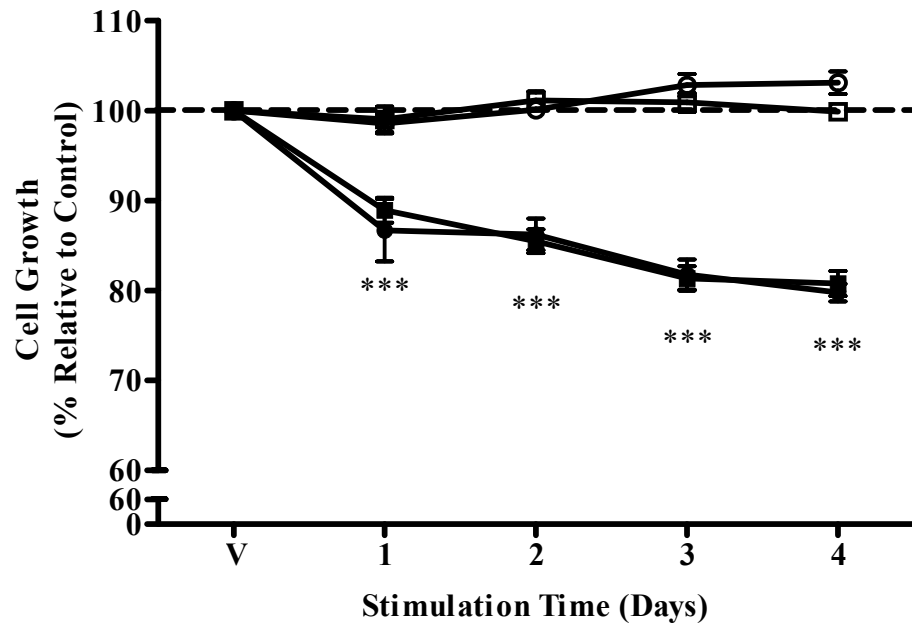


Figure 3.19: Sulforhodamine B assays depicting the BAPTA-AM insensitivity of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□, ○) or $G\alpha_q$ (■, ●) were incubated in media containing 10% serum in the presence (○, ●) or absence (□, ■) of 50 μ M BAPTA-AM prior to being treated with vehicle (0.2% propylene glycol; V) or 100 nM GnRH I for the indicated times. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls (for $G\alpha_q$ transfected cells).

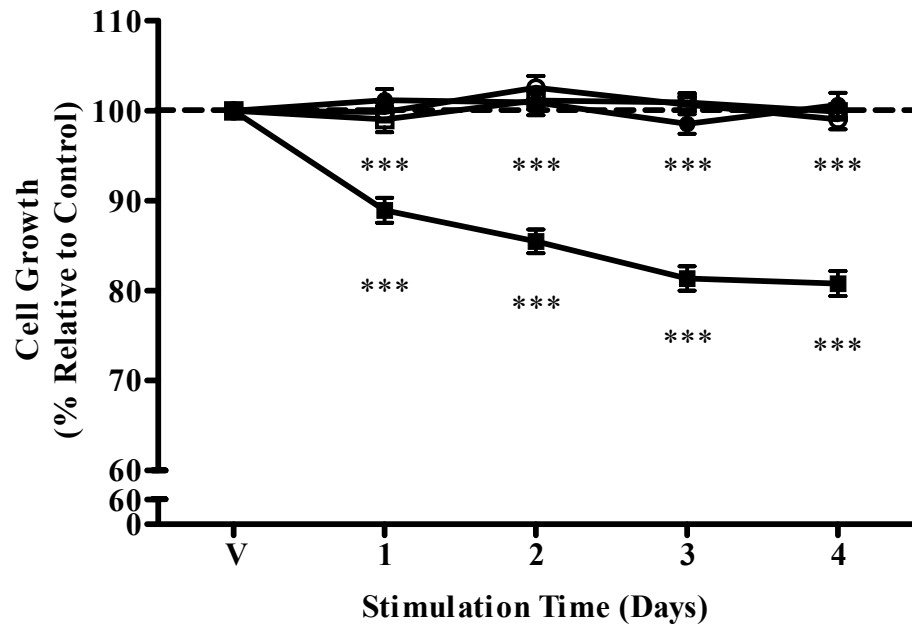


Figure 3.20: Sulforhodamine B assays depicting the PP2 sensitivity of the GnRH I-induced inhibition of cell growth. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently infected with GFP (□, ○) or $G\alpha_q$ (■, ●) were incubated in media containing 10% serum in the presence (○, ●) or absence (□, ■) of 10 μ M PP2 prior to being treated with vehicle (0.2% propylene glycol; V) or 100 nM GnRH I for the indicated times. Data from at least three independent experiments were quantified and the mean % cell growth \pm SE is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls and from GnRH I treated controls.

3.3 Discussion

Binding of GnRH to its receptor at the plasma membrane initiates a variety of intracellular signalling events with distinct physiological outcomes. Since the original observations of GnRH-induced cell growth inhibition on receptor expressing cell lines (Miller et al., 1985), substantial effort has been directed towards delineating the precise mechanism(s) involved. Considerable evidence suggests that the signalling cascades involved in GnRH-mediated cell growth inhibition are distinct from those involved in regulating gonadotropin secretion (Limonta et al., 2003, Maudsley et al., 2004, Millar et al., 2008) and one possibility to explain this divergent signalling is to hypothesise the inherent ability of the GnRH receptor to directly interact with multiple families of G proteins. Multiplicity of G protein coupling has after all been successfully confirmed for several other GPCRs including the β_2 -adrenergic receptor (Wenzel-Seifert and Seifert, 2000), the muscarinic receptors (Akam et al., 2001) and the chemokine CXCR₂ receptor (Hall et al., 1999). However, circumstantial evidence of such interaction forms the basis of much of the literature published to date and the frequently repeated paradigm of the ability of the GnRH receptor to directly interact with G_{i/o} and G_s has led to confusion regarding the mechanisms which underpin GnRH-mediated antiproliferation. To critically address this issue, I studied the G protein coupling profile of the GnRH receptor in stably transfected MEF cells derived from G $\alpha_{q/11}$ knockout mice (Offermanns et al., 1998). Additionally, I investigated the roles of the various G proteins in the activation of ERK, JNK and P38 and the downstream cellular effects of the activation of these MAPK modules.

GnRH-mediated MAPK activation has been extensively studied over the past two decades (Sim et al., 1993, Sim et al., 1995, Reiss et al., 1997, Haisenleder et al., 1998, Levi et al., 1998, Roberson et al., 1999, Naor et al., 2000, Benard et al., 2001, Bonfil et al., 2004, Davidson et al., 2004, Kraus et al., 2004, Maudsley et al., 2004, Dobkin-Bekman et al., 2006). Several investigations have shown that phosphorylation of these proteins readily occurs upon GnRH stimulation of receptor expressing cell lines and that this activation is dependent on the activity of PLC β and PKC. Indeed, Roberson and colleagues showed that specific depletion of PKC

isoforms in α T31 cells blocks the GnRH-induced activation of P38 (Roberson et al., 1999). Similarly, Levi and colleagues demonstrated that PKC inhibition with GF109203X in the same cell type is sufficient to significantly inhibit GnRH-mediated JNK activation (Levi et al., 1998). These studies thus suggest that GnRH-induced MAPK activation is, at least partially, dependent on $G_{q/11}$. However, the role of $G_{i/o}$ in MAPK activation in response to GnRH has also been the subject of much research. ERK activation in α T31 cells has been suggested to depend on a dual mechanism involving PKC and $G_{i/o}$ (Sim et al., 1995). Similarly, in Caov3 cells (which have been shown to express GnRH receptor mRNA), ERK activation has been hypothesised as being mediated by a combination of interactions involving the $G_{\alpha_{i/o}}$ G protein α subunits and the $G\beta\gamma$ complex (Kimura et al., 1999). Studies using PTX have also proposed a role for $G_{\alpha_{i/o}}$ in the GnRH-induced phosphorylation of JNK and P38 (Maudsley et al., 2004). Interestingly, my data contradicts these studies as I have found no evidence to support the theory of GnRH-induced activation of these MAPKs by $G_{i/o}$, even when artificially overexpressed in the $G_{\alpha_{q/11}}$ knockout MEF cell system. I have however confirmed that $G_{q/11}$ -mediated signalling plays a pivotal role.

Elegant studies have shown that the expression of $G_{\alpha_{q15}}$ with other GPCRs successfully enables $G_{i/o}$ -coupled receptors to signal through $PLC\beta$ and PKC (Conklin et al., 1993, Coward et al., 1999). It follows then that the inability of GnRH to induce phosphorylation of ERK, JNK or P38 when $G_{\alpha_{q15}}$ is expressed suggests that the GnRH receptor does not directly interact with the G protein α subunits of the $G_{i/o}$ family. This conclusion is strengthened by the observation that GnRH does not inhibit isoproterenol-induced cAMP accumulation. However, by definition, one of the most direct measures of G protein activation by an activated receptor is receptor catalysed GDP/GTP exchange. Other analytical approaches may yield indirect results and the notion of GnRH receptor- $G_{i/o}$ interaction has largely been derived from circumstantial evidence using PTX (Hawes et al., 1993, Sim et al., 1995, Imai et al., 1996, Kimura et al., 1999, Limonta et al., 1999, Maudsley et al., 2004, Romanelli et al., 2004, Imai et al., 2006). In this case, recent research from our laboratory (White et al., 2008a), taken together with the observations of Grosse

and colleagues who reported exclusive GnRH receptor-mediated labelling of $G_{\alpha_{q/11}}$ with [α - ^{32}P]GTP azidoanilide (Grosse et al., 2000), show definitively that the GnRH receptor does not directly couple to $G_{i/o}$ but does interact with $G_{q/11}$. A report by Shah and colleagues using activated G protein subunits as an output support this interpretation (Shah et al., 1995). Here, stimulation of αT31 cells with GnRH agonists resulted in the elevated agonist-induced downregulation of $G_{\alpha_{q/11}}$. $G_{\alpha_{i/o}}$ remained unaffected. Interestingly, it has recently been shown that the G protein α subunits can be activated directly by RTKs (Shan et al., 2006). Thus, it is possible that GnRH receptor-mediated RTK transactivation could be responsible for previous interpretations implicating a direct interaction of the receptor with $G_{i/o}$.

In the early 1990s, Janovick and Conn showed that treatment of rat pituitary cultures with CTX increased LH release in response to GnRH treatment and interpreted this finding as demonstrating that the GnRH receptor is directly coupled to G_s (Janovick and Conn, 1993). Since then, many other groups have studied this possibility but the results are inconclusive. The cAMP pathway is important for gonadotrope function in that it is thought to play a role in the regulation of LH β gene expression (Starzec et al., 1989, Horton and Halvorson, 2004). However, the involvement of G_s in this GnRH-induced signalling cascade is still debated. Using palmitoylation as a measure of G protein activation, it was suggested that there was a direct interaction between the GnRH receptor and G_s in rat pituitary cells (Stanislaus et al., 1998b) and this hypothesis was recapitulated in the L β T2 cell line using cell permeable peptides that uncouple G_{α_s} from the receptor (Liu et al., 2002b). However, the Gudermann group examined the possibility of a direct interaction between the activated receptor and G_{α_s} and, in agreement with my findings, failed to detect any G_{α_s} -mediated signal transduction in response to GnRH treatment (Grosse et al., 2000). Additionally, Larivière and colleagues recently identified a novel signalling pathway involving PKC δ and PKC ϵ which may mediate GnRH-induced activation of a cAMP sensitive promoter in L β T2 cells (Lariviere et al., 2007). These data, viewed collectively with my own observations, question the possibility of a direct interaction between the GnRH receptor and G_s . Interestingly, it has also been shown that specific adenylyl cyclase isoforms may be activated directly by the G $\beta\gamma$ complex (Gao and Gilman,

1991, Tang and Gilman, 1991, Yoshimura et al., 1996, Steiner et al., 2006) or independently of G proteins altogether (Feldman and Gros, 2007). Given that I cannot detect any cAMP accumulation in response to GnRH treatment in the $G\alpha_{q/11}$ knockout MEF cell model, taken together with the fact that the β_2 -adrenergic receptor agonist isoproterenol can elicit a marked increase in intracellular cAMP, I would suggest that the GnRH receptor does not directly interact with G_s .

The MAPK pathways are evolutionarily conserved kinase cascades that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis. Historically, ERK signalling was synonymous with cell proliferation (Dhillon et al., 2007) whilst the JNK and the P38 pathways were regarded as being stress activated (Yamauchi et al., 2001). Involvement of the JNK and the P38 signalling cascades in the GPCR-induced inhibition of cell proliferation has been widely documented (Pearson et al., 2001, Yamauchi et al., 2001, Dhillon et al., 2007, Raman et al., 2007) and here the GnRH receptor is no exception (Kraus et al., 2004, Maudsley et al., 2004, Dobkin-Bekman et al., 2006, Zhang et al., 2006). In contrast to these data, I showed that P38 and JNK do not influence GnRH I-induced cell growth inhibition. Additionally, I demonstrated that, in agreement with previous studies (Kimura et al., 1999, Kim et al., 2006), the ERK signalling pathway plays a critical role.

ERK has been implicated in cell growth inhibition, cell cycle arrest and the induction of proapoptotic signalling in a number of cell types (Goulet et al., 2005, Koike et al., 2006) and a large body of evidence indicates that the strength and duration of the ERK signal is kernel in determining cellular fate (Marshall, 1995, Meloche and Pouyssegur, 2007, White et al., 2008b). Several studies have shown that strong and prolonged activation of ERK by constitutively active Ras or Raf leads to arrest in the G_1 phase of the cell cycle by inducing the expression of cyclin-dependent kinase (CDK) inhibitors such as p53 and p21 (Pumiglia and Decker, 1997, Sewing et al., 1997). As such, it seems plausible to speculate that continuous exposure of the $G\alpha_{q/11}$ knockout cells to GnRH I would result in ERK activation of a similar strength and duration. Thus, by inhibiting this pathway, this activation is abolished and the

GnRH I-induced inhibition of cell growth is significantly diminished. Interestingly, Zhang and colleagues demonstrated that, in L β T2 cells, p53 is phosphorylated by GnRH in a P38-dependent manner (Zhang et al., 2006). While my results do not indicate a role for P38 in GnRH I-mediated cell growth inhibition, it is possible that the transformation of the L β T2 cell line with SV40 large T antigen may influence the signalling pathways involved. Indeed, the transformation domains of this oncoprotein have been shown to directly interact with a number of intracellular proteins including p53 (Ali and DeCaprio, 2001).

The observation that both G α_q and src were necessary for GnRH I to induce a significant inhibition of cell growth, but that this inhibition occurred independently of PKC and Ca²⁺, suggests that the GnRH receptor has the ability to modulate the activity of other G_{q/11}-dependent signalling pathways which regulate GnRH I-mediated antiproliferation. An attractive candidate which may be involved in such signalling events is SHP-2. Indeed, Coetsee and colleagues have demonstrated that GnRH I potently induces the activation of SHP-2 (Coetsee and Lu, unpublished observations). Furthermore, the intracellular domain of TM7 of the GnRH receptor possesses a YxxL motif which is known to, when Tyr phosphorylated, form part of the consensus sequence involved in the binding of SHPs to immunoreceptors (Unkeless and Jin, 1997). Additionally, SHP-2 has been implicated in the bradykinin-induced regulation of cellular proliferation at the B₂ receptor (Duchene et al., 2002) and both SHP-1 and SHP-2 have been demonstrated to be critical for the somatostatin SST₂ receptor-induced inhibition of cell growth to occur (Lopez et al., 2001, Ferjoux et al., 2003). Interestingly, Ferjoux and colleagues also showed that src is central to the initiation of the somatostatin-induced response (Ferjoux et al., 2003). Upon SST₂ receptor activation, src was demonstrated to Tyr phosphorylate the receptor protein (Ferjoux et al., 2003). Such phosphorylation results in SHP-2 recruitment to the newly formed SH2 binding domain, consequent activation of the phosphatase and subsequent somatostatin-induced cell growth inhibition (Ferjoux et al., 2003). Similarly, SHP-2 activation by GnRH has been shown to be totally dependent on src activation (Coetsee and Lu, unpublished observations). Thus, I propose that the GnRH-mediated activation of SHP-2 may be involved in the

regulation of the GnRH I-induced antiproliferative response. Furthermore, it is possible to speculate on the signalling pathways that may be important.

Firstly, analogous to the results obtained by Ferjoux and colleagues (Ferjoux et al., 2003) and Voisin and colleagues (Voisin et al., 2008), it is plausible that src may be activated by the $G\beta\gamma$ complex of $G_{q/11}$. Indeed, src has previously been shown to be activated downstream of these G protein subunits (Shajahan et al., 2004, Gentili et al., 2006). In this regard, the presence of $G\alpha_{q/11}$ would be necessary for dissociation of the heterotrimeric complex. Upon receptor activation and consequent dissociation, $G\beta\gamma$ -mediated src activation and resultant Tyr phosphorylation of the receptor at the YxxL motif could occur. Such signalling events could result in SHP-2 recruitment and activation and, consequently, downstream regulation of the ERK cascade. Indeed, Coetsee and colleagues have shown that a SHP-2 dominant negative construct significantly inhibits GnRH-induced ERK activation (Coetsee and Lu, unpublished observations) and my results indicate that the GnRH I-mediated activation of the ERK cascade is involved in the GnRH I stimulated inhibition of cell growth. In addition, evidence suggests that it is not possible to demonstrate GnRH-induced SHP-2 activation in the $G\alpha_{q/11}$ knockout MEF cell line implying that $G\alpha_{q/11}$ is necessary for such activation to occur (Coetsee and Lu, unpublished observations).

A second possibility to explain my observations is based on a study by Feng and colleagues (Feng et al., 2002). The authors demonstrated that SHP-1-AT₂ receptor association and consequent SHP-1 activation requires the presence of $G\alpha_s$ but is independent of both G protein activation ($G\alpha_s$ was shown to interact with SHP-1 and thus act as a scaffold for the phosphatase at this GPCR) and angiotensin-induced AT₂ receptor Tyr phosphorylation. Interestingly, however, the authors did not investigate the role of the Tyr residue in the NPxxY motif of the AT₂ receptor and this residue has been shown to be critical for SHP-2 activation via the B₂ receptor (Duchene et al., 2002). Thus, in the context of my data, upon transfection of $G\alpha_q$ and receptor activation, SHP-2 may be expected to associate with both the GnRH receptor and $G\alpha_{q/11}$. Such association may mediate GnRH I-induced antiproliferative signalling through the ERK cascade. This association and consequent signalling, however, may

still require Tyr phosphorylation of the GnRH receptor which may involve the GnRH I-mediated activation of src. How src could become activated by this GPCR is, in the context of these experiments, not clear.

The ability of many hormones and neurotransmitters to evoke diverse physiological and pathological responses, by binding to a single cognate receptor, brought about the hypothesis that one GPCR may have the inherent ability to activate multiple G proteins. As such, the differential effects of GnRH at central and peripheral sites have been proposed to be mediated via interaction of the activated receptor with $G_{q/11}$, $G_{i/o}$ and G_s . In this chapter, I have provided evidence to suggest that the GnRH receptor does not couple to G proteins of either the $G_{i/o}$ or the G_s families, that MAPK activation in response to GnRH treatment is entirely dependent, of the G protein α subunits tested, on $G\alpha_{q/11}$ and that the src and the ERK pathways are significantly involved in GnRH I-mediated cell death. Thus, while I cannot completely rule out cell background specific differences, I propose that signal transduction that is indicative of coupling to $G_{i/o}$ or G_s is determined downstream of the receptor-G protein interface or is mediated by different signalling mechanisms which have yet to be elucidated.

Chapter Four

GnRH Receptor Activation of the $G_{12/13}$

**G Protein Family and Support for the
Concept of Ligand-Induced Selective Signalling**

4.0 Abstract

Previous studies propose that the GnRH receptor may adopt multiple active conformations and modulate the activity of distinct intracellular signalling cascades. Furthermore, a large number of $G_{q/11}$ -coupled GPCRs are known to directly interact with $G_{12/13}$. In this chapter, given my previous data, I investigated the possibility of GnRH-induced activation of the $G_{12/13}$ G proteins. Transient transfection of both the $G\alpha_{12}$ and the $G\alpha_{13}$ subunits into $G\alpha_{q/11}$ knockout MEF cells resulted in $G_{q/11}$ -independent ERK activation. In BHK cells, which endogenously express both $G_{q/11}$ and $G_{12/13}$, GnRH I and GnRH II stimulated SRE-luciferase reporter gene activity. Both signals were significantly reduced by YM-254890 and $G\alpha_{13}$ ($G\alpha_{13}G225A$) and p115 RhoGEF (ΔDH -p115 RhoGEF) dominant negative mutants. Transient transfection of increasing amounts of $G\alpha_{13}G225A$ cDNA gradually decreased the GnRH stimulation of PhI hydrolysis. Additionally, the GnRH receptor and both endogenous $G\alpha_{13}$ and $G\alpha_{13}G225A$ could be coimmunoprecipitated. Surprisingly, stimulation of $G\alpha_{q/11}$ knockout MEF cells with GnRH I promoted the cortical polymerisation of actin and the formation of lamellipodia. In contrast, stimulation with GnRH II increased the formation of stress fibres. Y-27632, a ROCK inhibitor, potentiated the GnRH I-induced actin polymerisation and completely abolished the GnRH II stimulated response. A src inhibitor (PP2) markedly inhibited the GnRH I-induced cytoskeletal change but had no effect on the GnRH II stimulated stress fibre formation. Coimmunoprecipitation confirmed that the GnRH receptor could interact with src. In $G\alpha_{q/11}$ knockout MEF cells or SCL60 cells in the presence of YM-254890, GnRH I but not GnRH II elicited an increase in src Tyr phosphorylation. Interestingly, the transcriptional activity of a LH β and a FSH β promoter was partially inhibited by a PLC β inhibitor (U-73122) and ΔDH -p115 RhoGEF but not PP2. U-73122 and ΔDH -p115 RhoGEF together completely abolished the GnRH I stimulated response. These data therefore suggest that the GnRH receptor can couple to $G_{12/13}$ and that this signalling pathway may play an important role in the regulation of the synthesis of the gonadotropins. In addition, they confirm that the GnRH receptor may directly interact with src and suggest that GnRH I but not GnRH II may, independently of $G_{q/11}$, Tyr phosphorylate and thus activate this Tyr kinase.

4.1 Introduction

Evidence from our laboratory proposes that the GnRH receptor may adopt multiple receptor active conformations, which can be induced by the binding of different GnRH ligands, and thus differentially activate discrete intracellular signalling cascades. Lu and colleagues showed that individual Ala substitution of Met¹³², Met²²⁷, Phe²⁷², Phe²⁷⁶, Ile³²² and Tyr³²³, which lie deep within the TMs and are proposed to be remote from the ligand binding site, specifically increases the binding affinity of GnRH II but not GnRH I (Lu et al., 2005). These results therefore suggest that the binding of GnRH I and GnRH II facilitates the disruption of different combinations of receptor intramolecular constraints and thus induces different conformational changes of this GPCR. Given that the evidence presented within chapter three suggests that the GnRH receptor couples to $G_{q/11}$, but not to $G_{i/o}$ or G_s , these data propose that it may modulate the activity of other as yet undefined signalling pathways. Taken together with the fact that a large number of $G_{q/11}$ -coupled GPCRs are known to also activate $G_{12/13}$ (Riobo and Manning, 2005), I investigated the possible GnRH-mediated activation of the $G_{12/13}$ G protein family.

The potential activation of $G_{12/13}$ by GnRH has not been well elucidated. Grosse and colleagues were unable to detect interaction between this GPCR and $G\alpha_{12}$ or $G\alpha_{13}$ using [α -³²P]GTP azidoanilide and subsequent immunoprecipitation with antisera raised against these $G\alpha$ subunits (Grosse et al., 2000). However, their assays were carried out on plasma membrane preparations of α T31 and CHO cells. Recent evidence suggests that the $G\alpha_{13}$ subunits localise exclusively within the cytoplasm of COS7 cells and only translocate to the membrane upon GPCR activation (Yamazaki et al., 2005). Additionally, the cellular background in which any experiments are performed has been suggested to influence the stoichiometry of GnRH-induced signalling, in part by modulating the G protein coupling ability of the GnRH receptor (Dobkin-Bekman et al., 2006). Furthermore, competition among many different G proteins for a receptor may give rise to negative results (Riobo and Manning, 2005). As discussed in the previous chapter, expression of the GnRH receptor in $G\alpha_{q/11}$ knockout MEF cells eliminates the potential competition from $G_{q/11}$. Thus, the use of this cell model may better facilitate the identification of $G_{q/11}$ -independent signalling

pathways. In this chapter, using $G_{\alpha_{q/11}}$ knockout MEF cells and pathway specific inhibitors, dominant negative constructs and coimmunoprecipitation assays in other cell lines, I show that the GnRH receptor can directly activate $G_{12/13}$. Furthermore, the evidence gained during the completion of these studies confirms previous work from our laboratory indicating that the GnRH receptor may interact with src Tyr kinase and provides data to suggest that GnRH I but not GnRH II may induce $G_{q/11}$ -independent src activation.

4.2 Results

4.2.1 GnRH I Stimulates $G_{q/11}$ -Independent ERK Activation

The data presented within chapter three indicates that no GnRH-induced pERK1/2 response was evident in the $G_{\alpha_{q/11}}$ knockout MEF cell line which stably expresses the GnRH receptor. In order to eliminate the possibility that this result was due to a low endogenous cellular expression of $G_{12/13}$, or the sensitivity of the western detection system, I transiently transfected independently both $G_{\alpha_{12}}$ and $G_{\alpha_{13}}$ into these cells. Overexpression of $G_{\alpha_{12}}$ or $G_{\alpha_{13}}$ resulted in hitherto indemonstrable ERK activation in the absence of G_{α_q} . For cells transfected with $G_{\alpha_{12}}$, pERK1/2 was significantly maximally increased 1.9 ± 0.1 -fold above that of vehicle treated controls 5 minutes post-stimulation (figure 4.1). Similarly, $G_{\alpha_{13}}$ transfection elicited a significant maximal pERK1/2 response of 2.0 ± 0.0 -fold 5 minutes after GnRH I application. Consistent with my previous data, no significant increase in ERK activation was observed in vector transfected control cells at any time point tested.

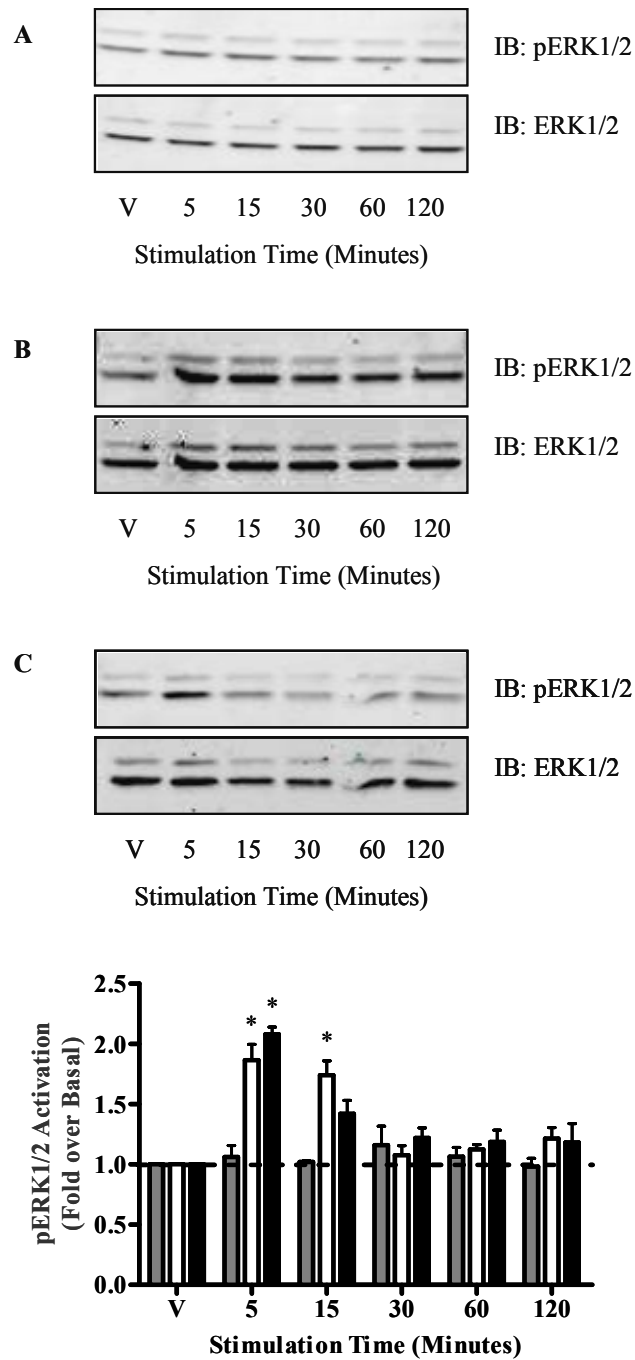


Figure 4.1 Immunoblots depicting the time dependence of the GnRH I-induced phosphorylation of ERK1/2. $G\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, transiently transfected with vector (A; grey bars), $G\alpha_{12}$ (B; white bars) or $G\alpha_{13}$ (C; black bars) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I for the indicated times. Representative blots are shown. Data from at least three independent experiments were quantified (using ERK1/2 as a loading control) and the mean fold over control \pm SE for the activation of ERK1/2 is presented. $p < 0.05$ (*) represents statistical significance from vehicle treated controls.

4.2.2 GnRH Stimulates SRE-Promoted Reporter Gene Activity

Arguably one of the most common methodologies used to study the activation of G proteins of the $G_{12/13}$ family is to observe the SRE-driven transcriptional response (Riobo and Manning, 2005). This technique does not provide definitive answers regarding direct GPCR-G protein interaction but it does provide an indication of GPCR ligand-induced $G_{12/13}$ activation. A number of studies have used constitutively active $G\alpha$ mutants to verify that $G_{12/13}$ stimulates SRE-promoted transcription (Fromm et al., 1997, Mao et al., 1998a, Shi et al., 2000, Suzuki et al., 2003, Vazquez-Prado et al., 2004). Similarly, I utilised GTPase deficient G protein α subunits to validate this method as a means of studying the possibility of GnRH-induced $G_{12/13}$ -mediated signalling. Only constitutively active $G\alpha_q$ ($G\alpha_qQ209L$), $G\alpha_{12}$ ($G\alpha_{12}Q231L$) or $G\alpha_{13}$ ($G\alpha_{13}Q226L$) could provoke significant increases in the SRE-luciferase signal and elicited responses of 2.7 ± 0.5 -fold, 1.5 ± 0.1 -fold and 2.2 ± 0.3 -fold that of vector transfected controls respectively (figure 4.2). $G\alpha_{i2}$ ($G\alpha_{i2}Q205L$) and $G\alpha_s$ ($G\alpha_sQ227L$) were completely without effect (1.0 ± 0.1 -fold and 1.0 ± 0.2 -fold respectively).

SRE-luciferase reporter gene assays revealed that both GnRH I and GnRH II were able to induce a SRE-driven transcriptional response. GnRH I elicited a significant increase in luciferase activity of 7.7 ± 0.3 -fold that of vehicle treated controls (figure 4.3; table 4.1). GnRH II, however, provoked a much lower luciferase response of 3.8 ± 0.3 -fold. These data are inconsistent with the GnRH-induced $G_{q/11}$ -mediated stimulation of PhI hydrolysis. In this case, both GnRH ligands stimulate a similar maximal activity (Lu et al., 2007). Dose-response data obtained after initial submission of this thesis also revealed that the luciferase output stimulated by both GnRH I and GnRH II is maximal at the $1 \mu M$ concentration used. Thus, in order to clarify the signalling cascades involved, signalling pathway specific inhibitors and dominant negative constructs were utilised. The transcriptional signal induced by both GnRH ligands was significantly inhibited by YM-254890 (GnRH I - 2.8 ± 0.1 -fold; GnRH II - 1.5 ± 0.1 -fold) (figure 4.4; table 4.1). Notably, however, the luciferase response elicited by either GnRH was still significantly increased above basal values and YM-254890 completely inhibits $G\alpha_{q/11}$ -mediated signalling at the

100 nM concentration used (figure 4.5). PTX had no significant effect on the luciferase readout obtained in response to either GnRH I or GnRH II (GnRH I - 8.4 ± 1.3 -fold; GnRH II - 3.4 ± 0.5 -fold) (figure 4.6; table 4.1). A dominant negative G α_{13} mutant (G α_{13} G225A) (GnRH I - 5.2 ± 0.2 -fold; GnRH II - 2.4 ± 0.1 -fold) (figure 4.7; table 4.1) and a dominant negative p115 RhoGEF mutant (Δ DH-p115 RhoGEF) (GnRH I - 5.3 ± 0.2 -fold; GnRH II - 2.1 ± 0.2 -fold) (figure 4.8; table 4.1) significantly inhibited the luciferase signals obtained after stimulation with either GnRH. Importantly, Δ DH-p115 RhoGEF has been demonstrated to inhibit the G α_{13} -induced SRE luciferase response but not that mediated by G α_q (Mao et al., 1998b). Interestingly, under all the conditions tested, a difference in the maximal SRE-luciferase signal induced by both GnRHs still existed. Transient expression of Δ DH-p115 RhoGEF and subsequent incubation with YM-254890 completely inhibited the luciferase response obtained after stimulation with GnRH II (1.0 ± 0.1 -fold) (figure 4.9; table 4.1). In contrast, GnRH I still displayed the ability to induce SRE-driven signalling and elicited a 1.8 ± 0.0 -fold increase in relative luciferase activity.

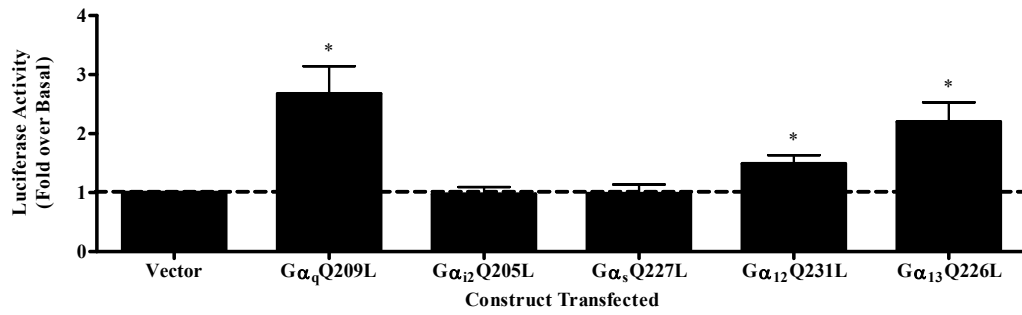


Figure 4.2 SRE-luciferase reporter gene assays depicting the GTPase deficient $G\alpha$ subunit-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing a SRE-luciferase reporter, transiently transfected with vector, $G\alpha_q$ Q209L, $G\alpha_{i2}$ Q205L, $G\alpha_s$ Q227L, $G\alpha_{12}$ Q231L or $G\alpha_{13}$ Q226L cDNA were serum starved for 40 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. $p < 0.05$ (*) represents statistical significance from vector transfected controls.

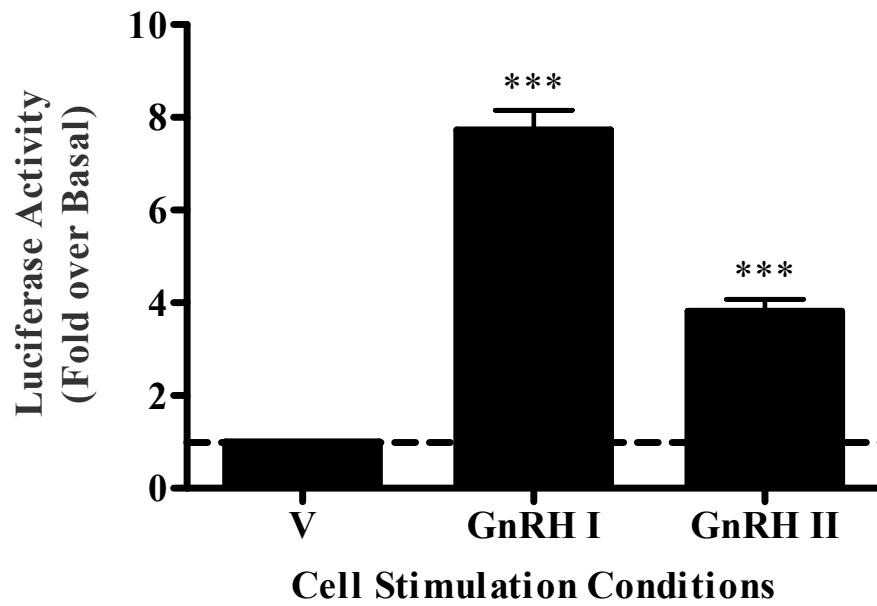


Figure 4.3 SRE-luciferase reporter gene assays depicting the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls.

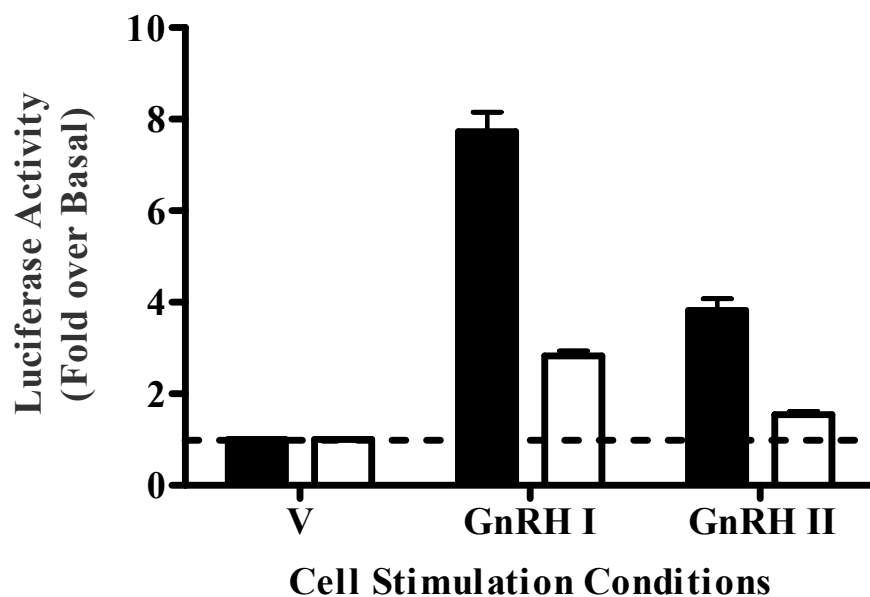


Figure 4.4 SRE-luciferase reporter gene assays depicting the YM-254890 sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 100 nM YM-254890 prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.1.

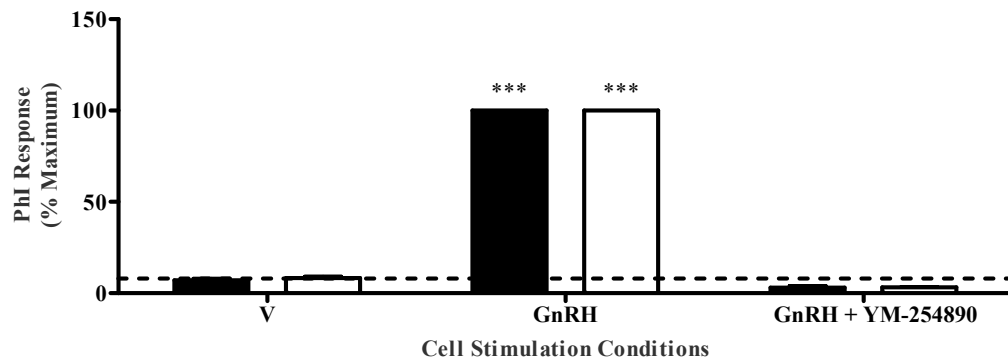


Figure 4.5 Phosphoinositide hydrolysis assays depicting the YM-254890 sensitivity of the GnRH-induced stimulation of phosphoinositide hydrolysis. BHK cells, stably expressing the GnRH receptor, were labelled overnight with 1 μ Ci/ml myo-D-[3 H]inositol in the presence or absence of 100 nM YM-254890 as indicated prior to being treated with 1 μ M GnRH I (black bars) or 1 μ M GnRH II (white bars) for 30 minutes. Data from at least three independent experiments were quantified and the mean % maximum \pm SE for phosphoinositide hydrolysis is presented. $p < 0.001$ (***) represents statistical significance from vehicle treated controls.

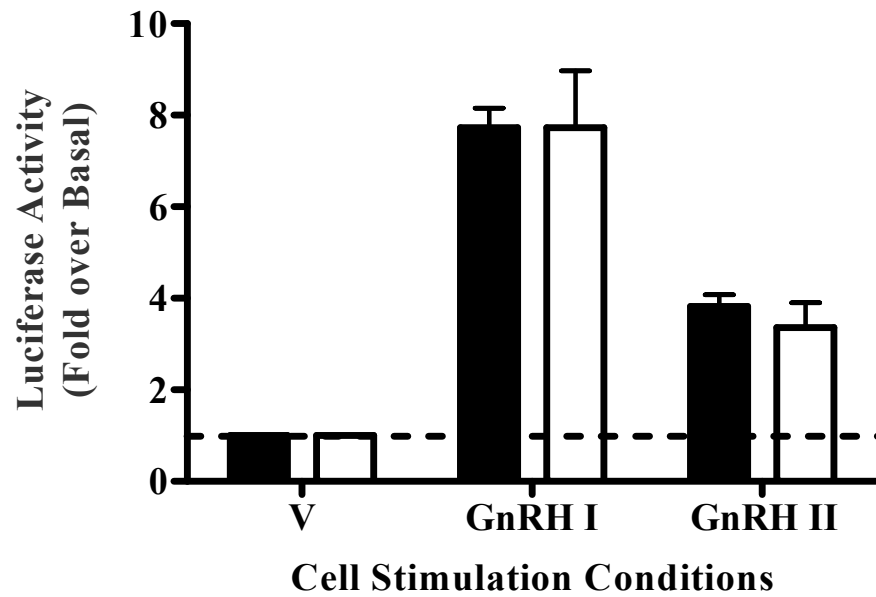


Figure 4.6 SRE-luciferase reporter gene assays depicting the PTX insensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 200 ng/ml PTX prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.1.

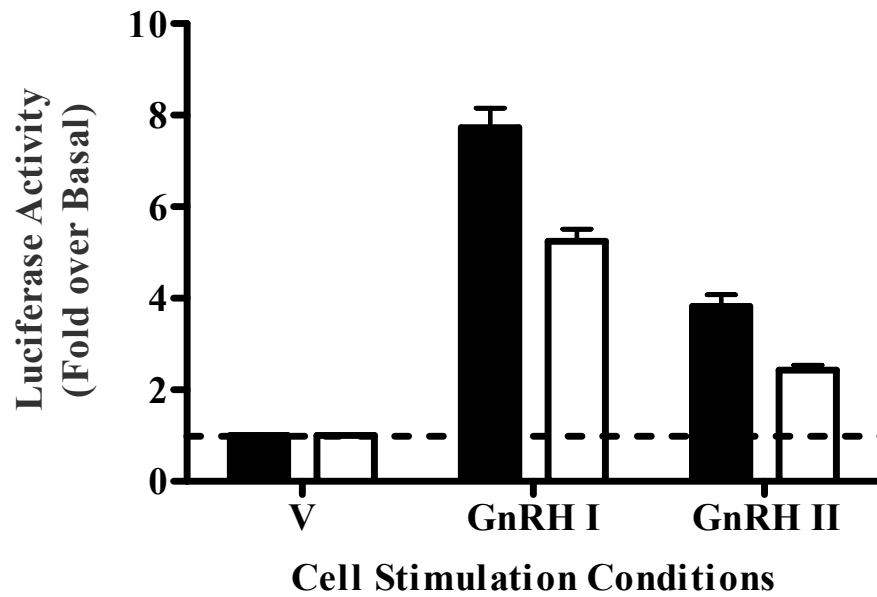


Figure 4.7 SRE-luciferase reporter gene assays depicting the $G\alpha_{13}G225A$ sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, transiently transfected with vector (black bars) or $G\alpha_{13}G225A$ (white bars) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.1.

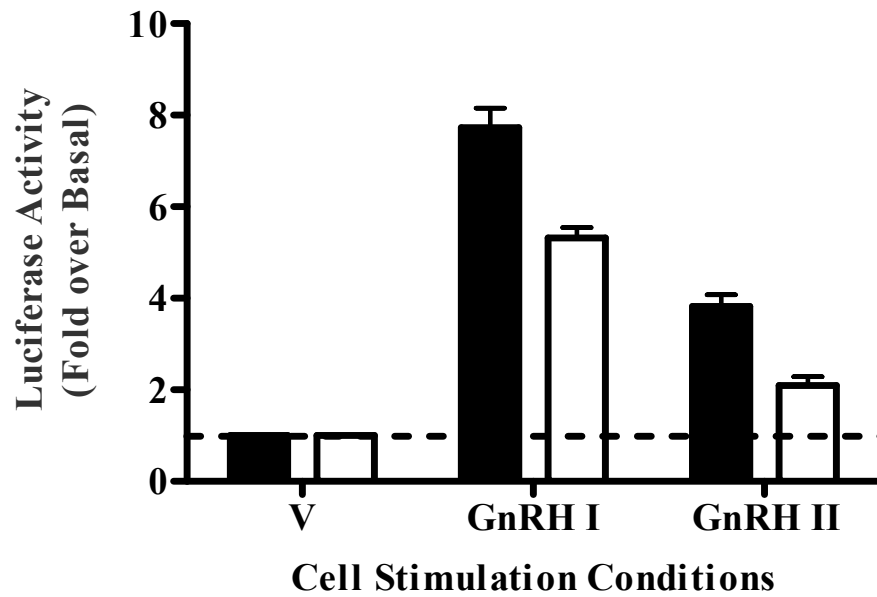


Figure 4.8 SRE-luciferase reporter gene assays depicting the Δ DH-p115 RhoGEF sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, transiently transfected with vector (black bars) or Δ DH-p115 RhoGEF (white bars) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.1.

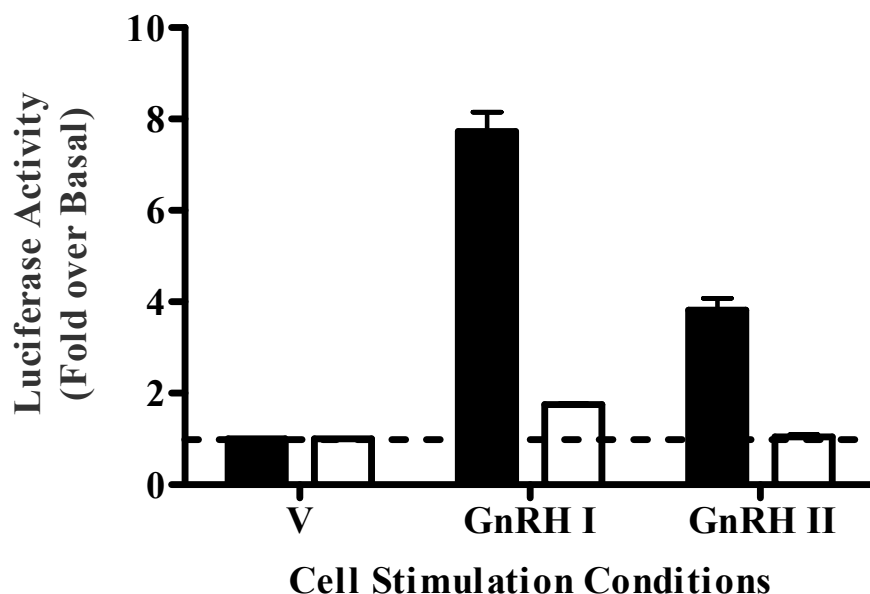


Figure 4.9 SRE-luciferase reporter gene assays depicting the Δ DH-p115 RhoGEF and YM-254890 sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, transiently transfected with vector (black bars) or Δ DH-p115 RhoGEF (white bars) cDNA were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 100 nM YM-254890 prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.1.

Chemical Inhibitor/Dominant Negative Construct	GnRH I		
	SRE-Luciferase Response (Fold)	Significant Increase Above Control? [†]	Significant Inhibition? [‡]
-	7.7 ± 0.3	***	-
YM-254890	2.8 ± 0.1	**	***
PTX	8.4 ± 1.3	**	ns
Gα ₁₃ G225A	5.2 ± 0.2	**	*
ΔDH-p115 RhoGEF	5.3 ± 0.2	**	*
ΔDH-p115 RhoGEF + YM-254890	1.8 ± 0.0	***	***

Chemical Inhibitor/Dominant Negative Construct	GnRH II		
	SRE-Luciferase Response (Fold)	Significant Increase Above Control? [†]	Significant Inhibition? [‡]
-	3.8 ± 0.3	***	-
YM-254890	1.5 ± 0.1	*	***
PTX	3.4 ± 0.5	*	ns
Gα ₁₃ G225A	2.4 ± 0.1	**	*
ΔDH-p115 RhoGEF	2.1 ± 0.2	*	*
ΔDH-p115 RhoGEF + YM-254890	1.0 ± 0.1	ns	**

Table 4.1 Statistical analysis of SRE-luciferase reporter gene assays depicting the chemical inhibitor and dominant negative construct (in)sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. Data from at least three independent experiments were quantified and the mean fold over control ± SE for the activation of SRE-luciferase is presented. *p*<0.05 (*), *p*<0.01 (**) and *p*<0.001 (***) represents statistical significance from vehicle treated controls ([†]) and from GnRH treated controls ([‡]).

4.2.3 The GnRH Receptor Directly Interacts with the $G_{12/13}$ G Proteins

Thus far I have provided evidence to suggest that GnRH may mediate $G_{12/13}$ -dependent signalling. These data, however, are based on distal phosphorylation and transcriptional signalling events and rely heavily on the use of chemical inhibitors. With this in mind, I sought to provide more upstream evidence to support my hypothesis that the GnRH receptor couples to $G_{12/13}$. Dominant negative constructs have previously been used in competitive binding experiments to elucidate the ability of a given GPCR to directly activate multiple G protein families. Indeed, a dominant negative $G\alpha_s$ mutant was sufficient to significantly inhibit the thyroid-stimulating hormone receptor-mediated stimulation of PhI hydrolysis by competing with native $G_{q/11}$ for activation by the receptor (Cleator et al., 2004). In contrast, it had no effect on the α_{1B} -adrenergic receptor-induced PhI response as this GPCR does not interact with G_s . These observations therefore support the proposition that, while not a direct measure, this technique gives a strong indication of interaction between a GPCR and specific G proteins. In the case of the GnRH receptor, transient transfection of increasing amounts of $G\alpha_{13}G225A$ significantly decreased the PhI response provoked by both GnRH I and GnRH II in a concentration-dependent manner (figure 4.10). Conversely, and in agreement with the data presented within chapter three, a $G\alpha_{i2}$ dominant negative ($G\alpha_{i2}G203T$) had no effect on the PhI response obtained after stimulation with either GnRH ligand.

$G\alpha_{13}G225A$ is thought to bind tightly to $G_{12/13}$ -coupled GPCRs in order to exert a dominant negative phenotype (Gohla et al., 1999, Orth et al., 2005). Indeed, analogous mutation of $G\alpha_s$ ($G\alpha_sG226A$) results in a G protein subunit mutant that is incapable of undergoing the conformational change necessary for GTP-induced G protein α subunit dissociation from the $G\beta\gamma$ complex (Miller et al., 1988, Lee et al., 1992b). Thus, I hypothesised that the GnRH receptor may form a stable complex with this mutant $G\alpha$ subunit such that constitutive physical association between the receptor and $G\alpha_{13}G225A$ should be detectable. Several studies have used coimmunoprecipitation in order to elucidate direct GPCR-G protein interaction. However, an inherent difficulty with such an approach is the transient nature of these contacts. In agreement with this, previous reports reveal that coimmunoprecipitation

of the β_2 -adrenergic receptor (Lachance et al., 1999) and the angiotensin AT₂ receptor (Feng et al., 2002) with G α_s allows only the weakest of signals corresponding to this α subunit to be detected. Such fundamental experimental problems may be overcome by utilising a dissociation defective mutant G α subunit. Indeed, as dissociation of the heterotrimeric complex is prevented in response to basal receptor signalling, direct GPCR-G protein interaction should become more readily detectable. Cotransfection of the human influenza hemagglutinin (HA) tagged GnRH receptor and G α_{13} G225A into COS7 cells revealed that both proteins could be coimmunoprecipitated in an easily detectable manner (figure 4.11). Vector transfected controls reinforced the specificity of this detection method as no specific bands were present in this lane. Additionally, in agreement with the studies discussed previously, a weak band corresponding to endogenous G α_{13} was present in samples transfected with only the HA tagged GnRH receptor. These data, viewed collectively, strongly suggest that the GnRH receptor directly interacts with G_{12/13}.

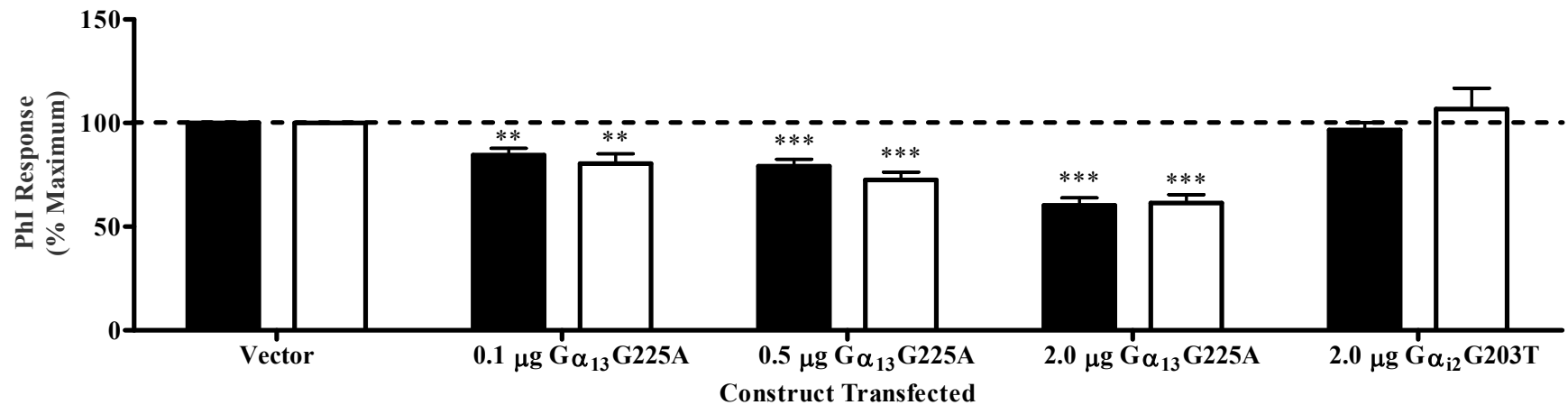


Figure 4.10 Phosphoinositide hydrolysis assays depicting the Gα₁₃G225A sensitivity of the GnRH-induced stimulation of phosphoinositide hydrolysis. BHK cells, stably expressing the GnRH receptor, transiently transfected with vector, 0.1 µg/well Gα₁₃G225A, 0.5 µg/well Gα₁₃G225A, 2.0 µg/well Gα₁₃G225A or 2.0 µg/well Gα₁₂G203T cDNA were labelled overnight with 1 µCi/ml myo-D-[³H]inositol prior to being treated with 1 µM GnRH I (black bars) or 1 µM GnRH II (white bars) for 30 minutes. The amount of cDNA transfected remained constant. Data from at least three independent experiments were quantified and the mean % maximum ± SE for phosphoinositide hydrolysis is presented. *p*<0.01 (**) and *p*<0.001 (***) represents statistical significance from GnRH treated controls.

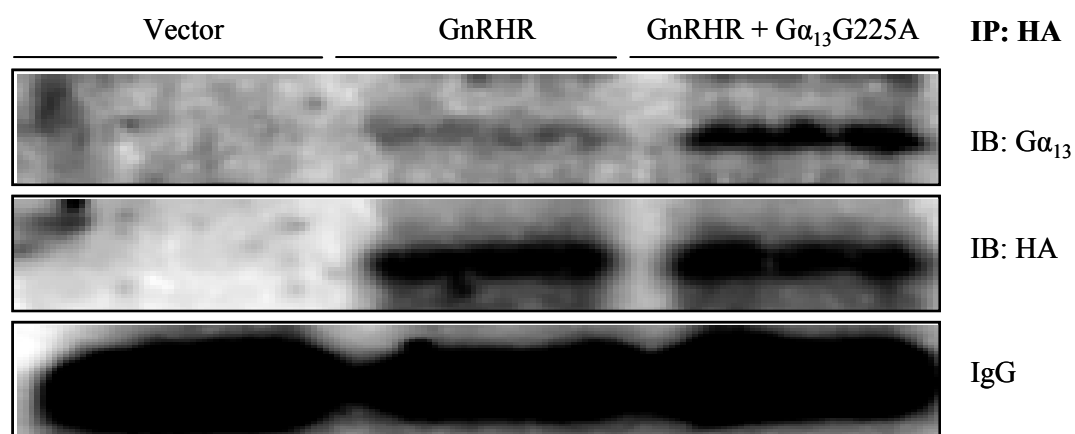


Figure 4.11 Coimmunoprecipitation assays depicting a direct interaction between the GnRH receptor and $G\alpha_{13}$. COS7 cells, transiently transfected with vector, HA tagged GnRH receptor or HA tagged GnRH receptor and $G\alpha_{13}$ G225A cDNA were serum starved for 16 hours prior to being lysed and incubated with an anti-HA agarose conjugate slurry with constant agitation at 4°C for 16 hours. The amount of cDNA transfected remained constant. Representative blots are shown.

4.2.4 GnRH Stimulates Cytoskeletal Reorganisation

A number of studies indicate that the $G_{12/13}$ G proteins can mediate actin stress fibre formation in a Rho-dependent manner (Buhl et al., 1995, Gohla et al., 1999). Additionally, evidence suggests that they are not involved in the positive regulation of Rac (Gratacap et al., 2001). In order to investigate the possibility of GnRH-mediated $G_{q/11}$ -independent actin reorganisation, I studied the cytoskeletal rearrangement induced by GnRH in the $G\alpha_{q/11}$ knockout MEF cell model. Following stimulation with either GnRH I or GnRH II, marked changes in cellular phenotype relative to that of vehicle treated controls were observed. β -actin immunocytochemical studies revealed that GnRH I induced both actin polymerisation at the cell cortex and lamellipodia formation (figure 4.12), both of which are accepted indicators of the activation of Rac (Ridley et al., 1992). In contrast, GnRH II stimulated stress fibre formation, a marker of Rho activation (Ridley and Hall, 1992). In an effort to elucidate the signalling cascades involved, pathway specific inhibitors were utilised. Microinjection of the ROCK catalytic domain into Swiss 3T3 cells has been shown to result in the formation of stress fibres (Amano et al., 1997). Additionally, ROCK has previously been demonstrated to be central to the $G_{12/13}$ -Rho-dependent induction of stress fibre formation (LePage et al., 2003). Y-27632, a ROCK inhibitor, completely inhibited the GnRH II-induced response. In contrast, it had the opposite effect on that elicited by GnRH I. In this case, the GnRH I stimulated actin polymerisation and lamellipodia formation were potentiated. Indeed, increased β -actin staining at the cell periphery and more pronounced lamellipodial extensions were apparent.

The observation that GnRH I induced Rac activation is consistent with a previous study (intriguingly, however, it is inconsistent with the $G_{12/13}$ -mediated activation of Rho). Davidson and colleagues demonstrated that GnRH I induced the alteration of cellular morphology and the *de novo* generation of polymerised actin structures (Davidson et al., 2004). Furthermore, the authors suggested that this effect was independent of the classical GnRH receptor- $G_{q/11}$ signalling pathway. Specific inhibition of src with PP2 was sufficient to completely prevent the GnRH I-induced response. How the GnRH I-bound GnRH receptor activated Rac remained

undefined. These data, taken together with the now established link between src and Rac (Servitja et al., 2003, Kawakatsu et al., 2005, Zhou et al., 2007, Dise et al., 2008) and the fact that previous work from our laboratory indicates that the GnRH receptor may interact with src (Coetsee and Lu, unpublished observations), prompted me to investigate the possible involvement of this Tyr kinase in the GnRH-mediated regulation of the actin cytoskeleton. Inhibition of src with PP2 resulted in a marked induction of actin stress fibre formation after stimulation with GnRH I (figure 4.11). In contrast, PP2 application had no effect on the GnRH II-induced response. Application of both Y-27632 and PP2 prevented any apparent change in the localisation or intensity of β -actin staining in response to either GnRH ligand.

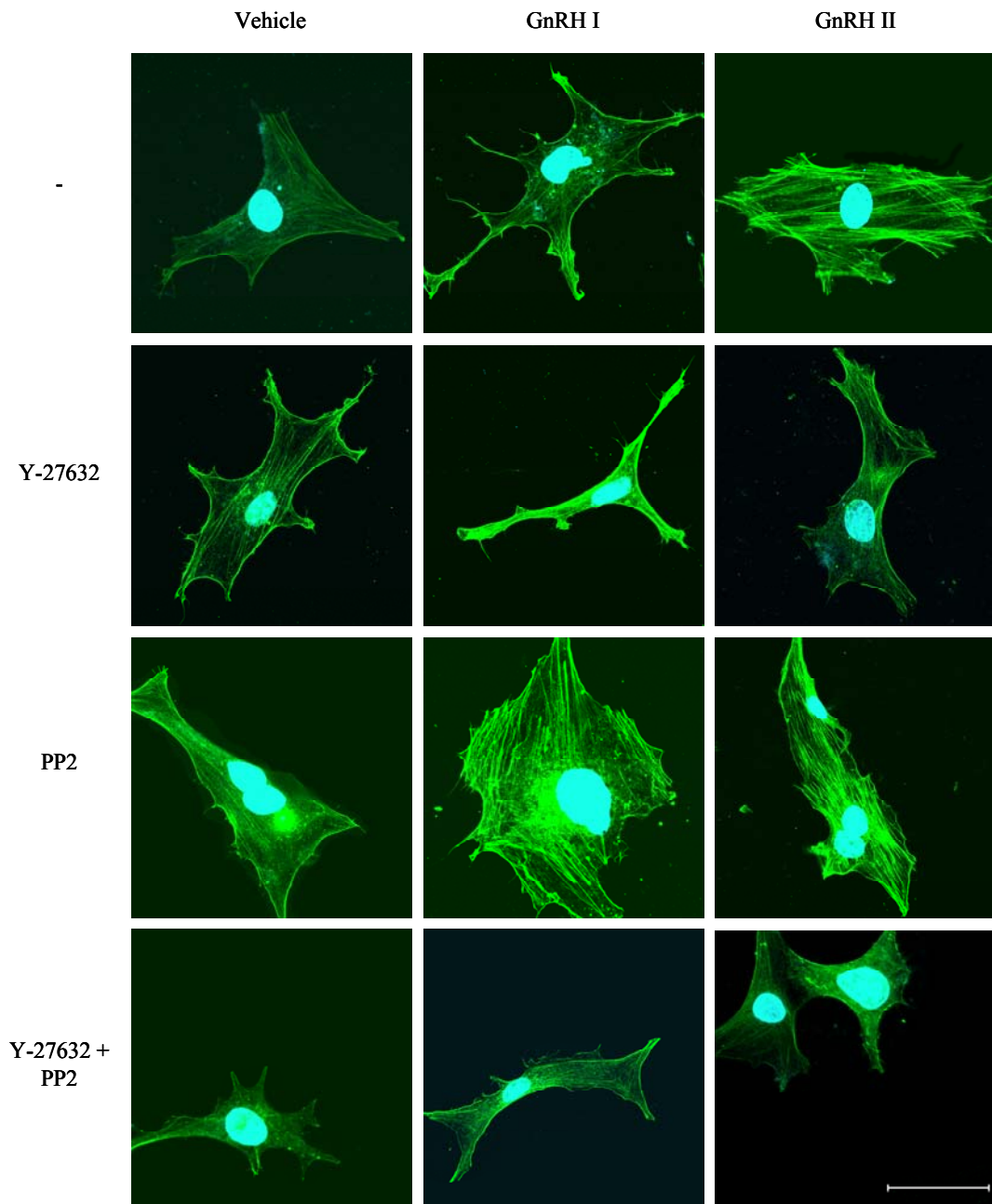


Figure 4.12 Immunocytochemistry depicting the Y-27632 and the PP2 sensitivity of the GnRH-induced alteration in the organisation of the actin cytoskeleton. $G_{\alpha_{q/11}}$ knockout MEF cells, stably expressing the GnRH receptor, were serum starved for 16 hours in the presence or absence of 25 μ M Y-27632, 10 μ M PP2 or 25 μ M Y-27632 and 10 μ M PP2 prior to being treated with vehicle (0.2% propylene glycol), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 60 minutes. Scale bar represents 50 μ M. Representative images are shown.

4.2.5 **GnRH I but not GnRH II Directly Activates Src Independently of $G_{q/11}$**

The immunocytochemical data described previously suggests that src is, independently of $G_{q/11}$, involved in the activation of Rac in response to GnRH I but not GnRH II. Indeed, inhibition of src with PP2 was sufficient to completely prevent the GnRH I-induced cortical localisation of actin and the formation of lamellipodia. In contrast, PP2 application had no effect on the response stimulated by GnRH II. Others within our group have previously shown that the GnRH receptor may interact with src (Coetsee and Lu, unpublished observations) and, initially, I sought to confirm these results. Transient transfection of the HA tagged GnRH receptor into COS7 cells revealed that both the receptor protein and src could be coimmunoprecipitated (figure 4.13). No specific bands were present in controls transfected with vector only.

Src activation events enable autophosphorylation of a Tyr residue within the kinase domain of the protein (Boggon and Eck, 2004). To investigate whether GnRH I and GnRH II could mediate the $G_{q/11}$ -independent activation of src, and in keeping with the work of Davidson and colleagues (Davidson et al., 2004), I examined src activation in SCL60 cells in the presence and absence of YM-254890. Prior to commencing these studies, I attempted to analyse the GnRH-induced src activation in the $G_{\alpha_{q/11}}$ knockout MEF cell model. In this cell line, however, significant Tyr phosphorylation of src was undetectable in response to either GnRH ligand. The western blots subjectively indicate that, relative to vehicle treated controls, src is Tyr phosphorylated only in response to GnRH I. The data sets are not, however, statistically significant when compared to either each either or cells treated only with vehicle (GnRH I - 1.8 ± 0.4 -fold; GnRH II - 1.2 ± 0.3 -fold) (figure 4.14). Nevertheless, these results do not preclude the above hypothesis as the sensitivity of the assay with regards to this cell line may be solely responsible. The use of the SCL60 cellular background also provides a positive control allowing confirmation of previous observations which suggest that src may be activated downstream of $G_{q/11}$ (Nagao et al., 1998) and therefore by both GnRH ligands. Stimulation with both GnRH I and GnRH II induced significant increases in pY⁴¹⁶-src giving responses of

2.8 ± 0.2 -fold and 2.4 ± 0.2 -fold that of vehicle treated controls respectively (figure 4.15; table 4.2). Specific inhibition of the $G\alpha_{q/11}$ signalling pathway with YM-254890 was sufficient to significantly reduce the levels of pY⁴¹⁶-src detected in response to both GnRHs. When compared to vehicle treated controls, however, a significant increase in phosphorylated src could still be detected in response to stimulation with GnRH I but not with GnRH II (GnRH I - 1.5 ± 0.1 -fold; GnRH II - 1.1 ± 0.1 -fold).

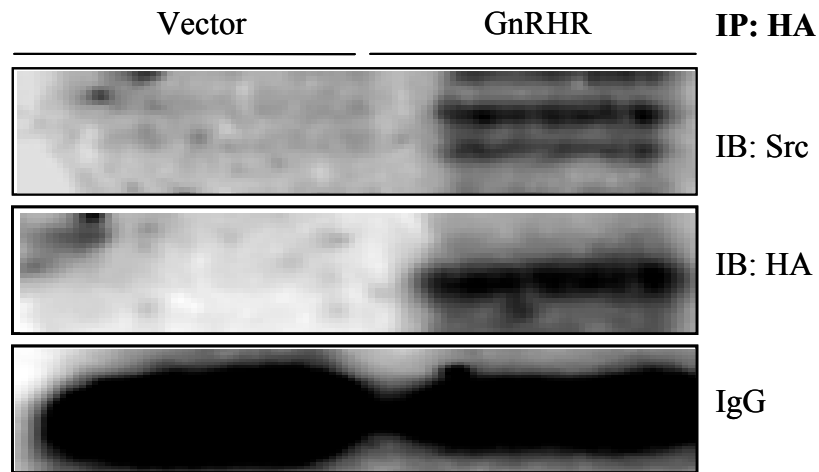


Figure 4.13 Coimmunoprecipitation assays depicting an interaction between the GnRH receptor and src. COS7 cells, transiently transfected with vector (lane 1) or HA tagged GnRH receptor (lane 2) cDNA were serum starved for 16 hours prior to being lysed and incubated with an anti-HA agarose conjugate slurry with constant agitation at 4°C for 16 hours. Representative blots are shown.

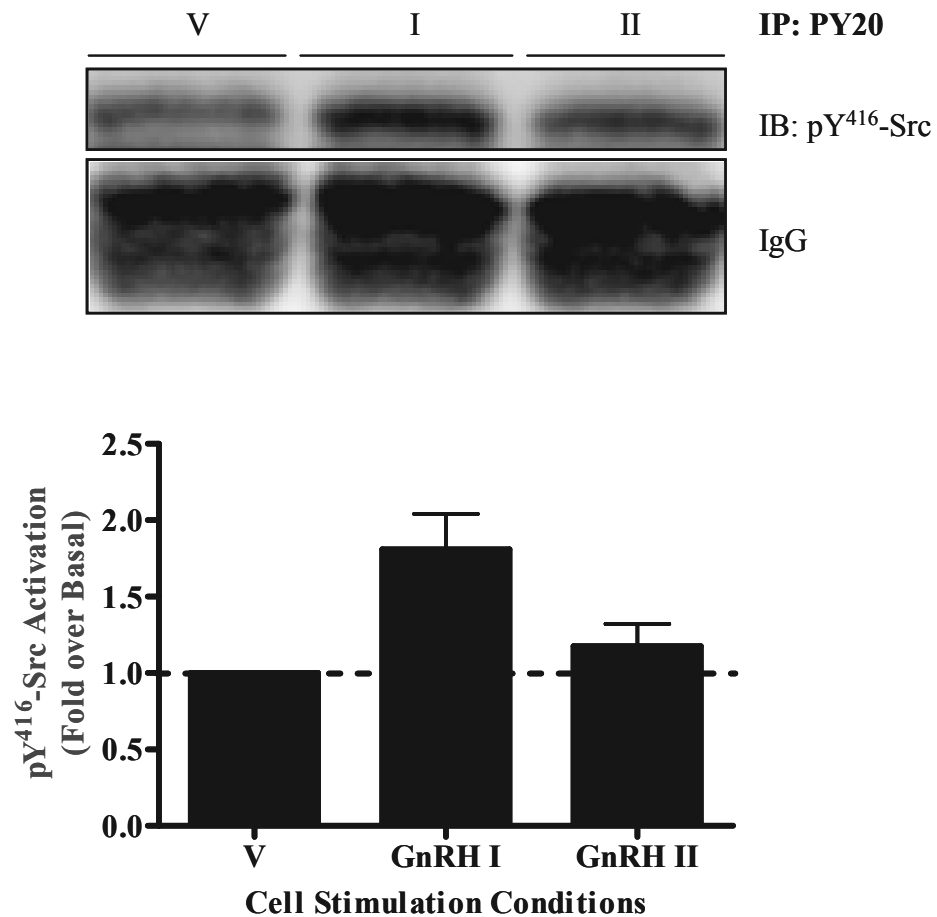


Figure 4.14 Immunoblots depicting the GnRH-induced phosphorylation of src. G $\alpha_{q/11}$ knockout MEF cells, stably expressing the GnRH receptor, were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I (I) or 1 μ M GnRH II (II) as indicated for 10 minutes. Representative blots are shown. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of pY⁴¹⁶-src is presented.

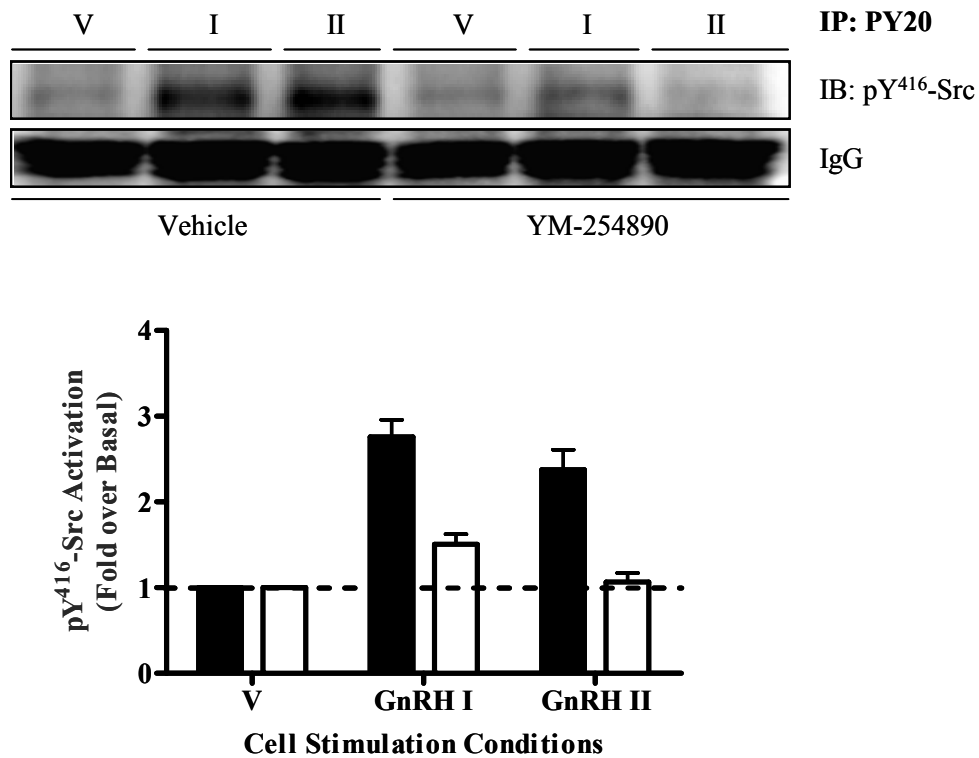


Figure 4.15 Immunoblots depicting the YM-254890 sensitivity of the GnRH-induced phosphorylation of src. SCL60 cells, stably expressing the GnRH receptor, were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 100 nM YM-254890 prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I (I) or 1 μ M GnRH II (II) as indicated for 10 minutes. Representative blots are shown. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of pY⁴¹⁶-src is presented. For ease of presentation, statistical analysis is shown in table 4.2.

GnRH Ligand	pY ⁴¹⁶ -src Activation (Fold)	Significant Increase Above Control? [†]	Significant Inhibition with YM-254890? [‡]	Significant Increase Above Control with YM-254890? [†]
GnRH I	2.8 \pm 0.2	*	**	*
GnRH II	2.4 \pm 0.2	*	**	ns

Table 4.2 Statistical analysis of immunoblots depicting the U-73122 sensitivity of the GnRH-induced phosphorylation of src. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the phosphorylation of src is presented. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) represents statistical significance from vehicle treated controls ([†]) and from GnRH treated controls ([‡]).

SRE-luciferase assays described earlier in this chapter demonstrated that GnRH I and GnRH II have the ability to induce significantly different levels of SRE-driven transcriptional activity (figure 4.3; table 4.1). Additionally, the data recently illustrated propose that src may associate with the GnRH receptor and may be, independently of $G_{q/11}$, activated by GnRH I. Furthermore, they indicate that src activation may subsequently be involved in the activation of Rac. Interestingly, constitutively active Rac has been shown to potently activate SRE-luciferase (Kim and Kim, 1997). These data therefore suggest that the difference in the SRE-luciferase response induced by both GnRH ligands may be due to interaction of the GnRH receptor with src and consequent GnRH I-induced, $G_{q/11}$ -independent, stimulation of Rac activity. In line with this proposal, inhibition of src with PP2 significantly decreased the SRE-mediated transcriptional signal in response to GnRH I but not to GnRH II (GnRH I - 5.3 ± 0.2 -fold; GnRH II - 3.3 ± 0.4 -fold) (figure 4.16; table 4.3).

I have previously shown that transient transfection of Δ DH-p115 RhoGEF and subsequent inhibition with YM-254890 completely inhibited the GnRH II-induced transcriptional signal (1.0 ± 0.1 -fold) (figure 4.9; table 4.1). In contrast, that mediated by GnRH I remained significantly increased above vehicle treated controls (1.8 ± 0.0 -fold). To investigate if src is involved in the residual GnRH I-induced signal, the effect of PP2 application was analysed. Transient transfection of Δ DH-p115 RhoGEF and subsequent inhibition with both YM-254890 and PP2 was sufficient to completely inhibit the SRE-driven transcription induced by stimulation with either GnRH ligand (GnRH I - 0.9 ± 0.1 -fold; GnRH II - 1.1 ± 0.1 -fold) (figure 4.17; table 4.3).

At the $G_{q/11}$ signalling pathway, both GnRHs are known to induce similar maximal responses (Lu et al., 2007). Consistent with these observations, inhibition of both the $G\alpha_{13}$ -p115 RhoGEF- and the src-mediated signalling pathways revealed that GnRH I and GnRH II were able to induce SRE-luciferase responses of similar levels (GnRH I - 2.6 ± 0.1 -fold; GnRH II - 2.3 ± 0.2 -fold) (figure 4.18; table 4.3). Additionally, no significant difference existed between the GnRH II-induced SRE-mediated

transcriptional response in cells inhibited with Δ ADH-p115 RhoGEF or Δ ADH-p115 RhoGEF and PP2 together (2.1 ± 0.2 -fold and 2.3 ± 0.2 -fold respectively). These data, viewed collectively, propose that GnRH I but not GnRH II may, independently of $G_{q/11}$, stimulate the Tyr phosphorylation and thus the activation of src.

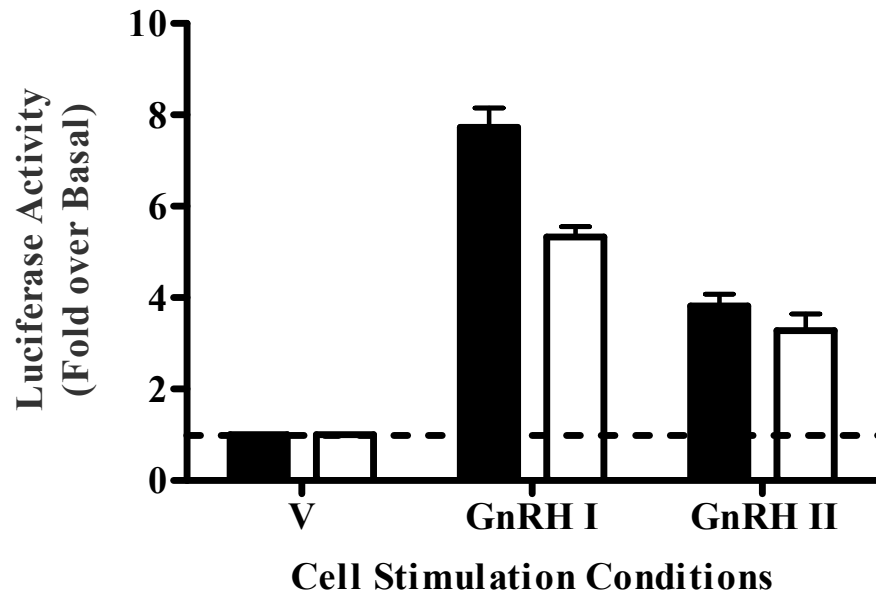


Figure 4.16 SRE-luciferase reporter gene assays depicting the PP2 sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 10 μ M PP2 prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.3.

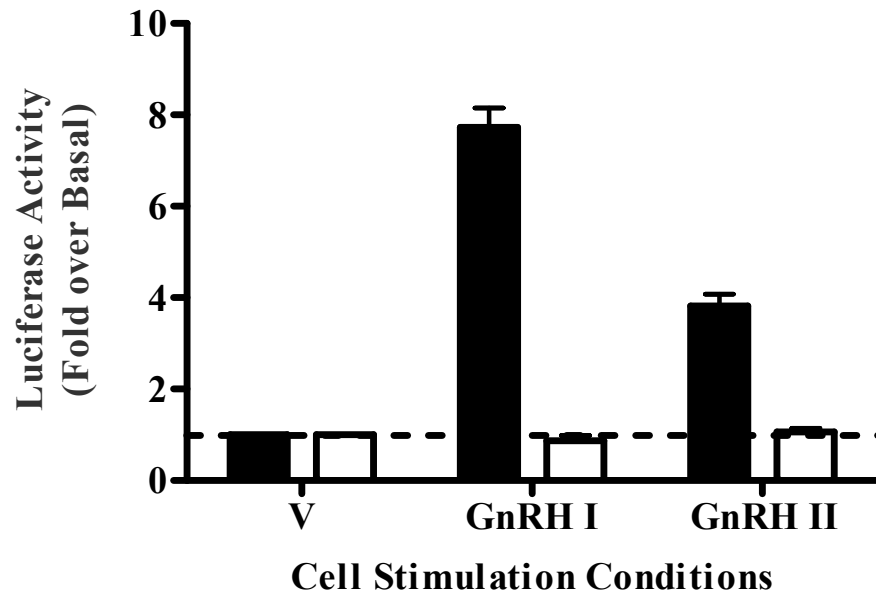


Figure 4.17 SRE-luciferase reporter gene assays depicting the Δ DH-p115 RhoGEF, YM-254890 and PP2 sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, transiently transfected with vector (black bars) or G α_{13} G225A (white bars) cDNA were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 100 nM YM-254890 and 10 μ M PP2 prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.3.

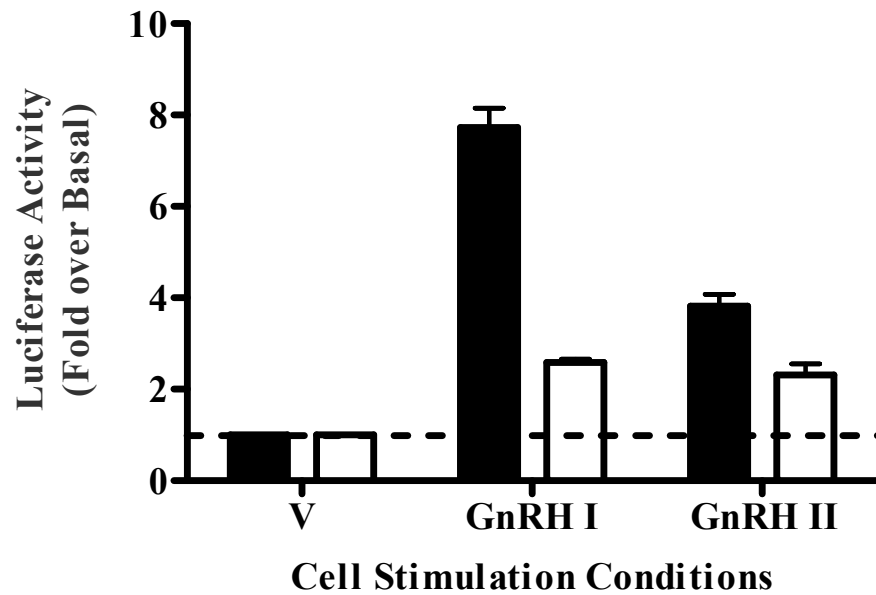


Figure 4.18 SRE-luciferase reporter gene assays depicting the Δ ADH-p115 RhoGEF and PP2 sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. BHK cells, stably expressing the GnRH receptor and a SRE-luciferase reporter, transiently transfected with vector (black bars) or G α_{13} G225A (white bars) cDNA were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 10 μ M PP2 prior to being treated with vehicle (0.2% propylene glycol; V), 1 μ M GnRH I or 1 μ M GnRH II as indicated for 24 hours. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of SRE-luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.3.

Chemical Inhibitor/Dominant Negative Construct	GnRH I		
	SRE-Luciferase Response (Fold)	Significant Increase Above Control? [†]	Significant Inhibition? [‡]
-	7.7 ± 0.3	***	-
PP2	5.3 ± 0.2	**	*
ΔDH-p115 RhoGEF + YM-254890 + PP2	0.9 ± 0.1	ns	***
ΔDH-p115 RhoGEF + PP2	2.6 ± 0.1	**	***
Chemical Inhibitor/Dominant Negative Construct	GnRH II		
	SRE-Luciferase Response (Fold)	Significant Increase Above Control? [†]	Significant Inhibition? [‡]
-	3.8 ± 0.3	***	-
PP2	3.3 ± 0.4	*	ns
ΔDH-p115 RhoGEF + YM-254890 + PP2	1.1 ± 0.1	ns	**
ΔDH-p115 RhoGEF + PP2	2.3 ± 0.2	*	***

Table 4.3 Statistical analysis of SRE-luciferase reporter gene assays depicting the chemical inhibitor and dominant negative construct (in)sensitivity of the GnRH-induced stimulation of SRE-promoted transcriptional activity. Data from at least three independent experiments were quantified and the mean fold over control ± SE for the activation of SRE-luciferase is presented. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) represents statistical significance from vehicle treated controls ([†]) and from GnRH treated controls ([‡]).

4.2.6 $G_{12/13}$ is Involved in LH β and FSH β Gene Transcription

In an effort to clarify a physiological role for these novel GnRH-induced signalling pathways, I investigated their possible involvement in GnRH stimulated LH β and/or FSH β gene transcription. GnRH I elicited significant increases in LH β - and FSH β -promoted luciferase activity (LH β - 20.9 ± 3.1 -fold; FSH β - 3.7 ± 0.3 -fold) (figure 4.19; table 4.4). The luciferase response obtained in both cases was significantly decreased, although not to basal levels, after inhibition of PLC β with U-73122 (LH β - 7.4 ± 0.3 -fold; FSH β - 1.9 ± 0.0 -fold) (figure 4.20; table 4.4) or transient transfection of Δ DH-p115 RhoGEF (LH β - 11.7 ± 1.9 -fold; FSH β - 1.8 ± 0.1 -fold) (figure 4.21; table 4.4). Transient transfection of Δ DH-p115 RhoGEF and subsequent incubation with U-73122 was sufficient to completely inhibit both luciferase signals obtained after GnRH I stimulation (LH β - 0.9 ± 0.1 -fold; FSH β - 0.9 ± 0.1 -fold) (figure 4.22; table 4.4). Additionally, PP2 was completely without effect (LH β - 19.4 ± 4.5 -fold; FSH β - 3.8 ± 0.4 -fold) (figure 4.23; table 4.4). These results suggest that GnRH-induced $G_{12/13}$ -mediated signalling but not GnRH-induced $G_{q/11}$ -independent src-mediated signalling may be involved in the transcriptional regulation of these gonadotropins.

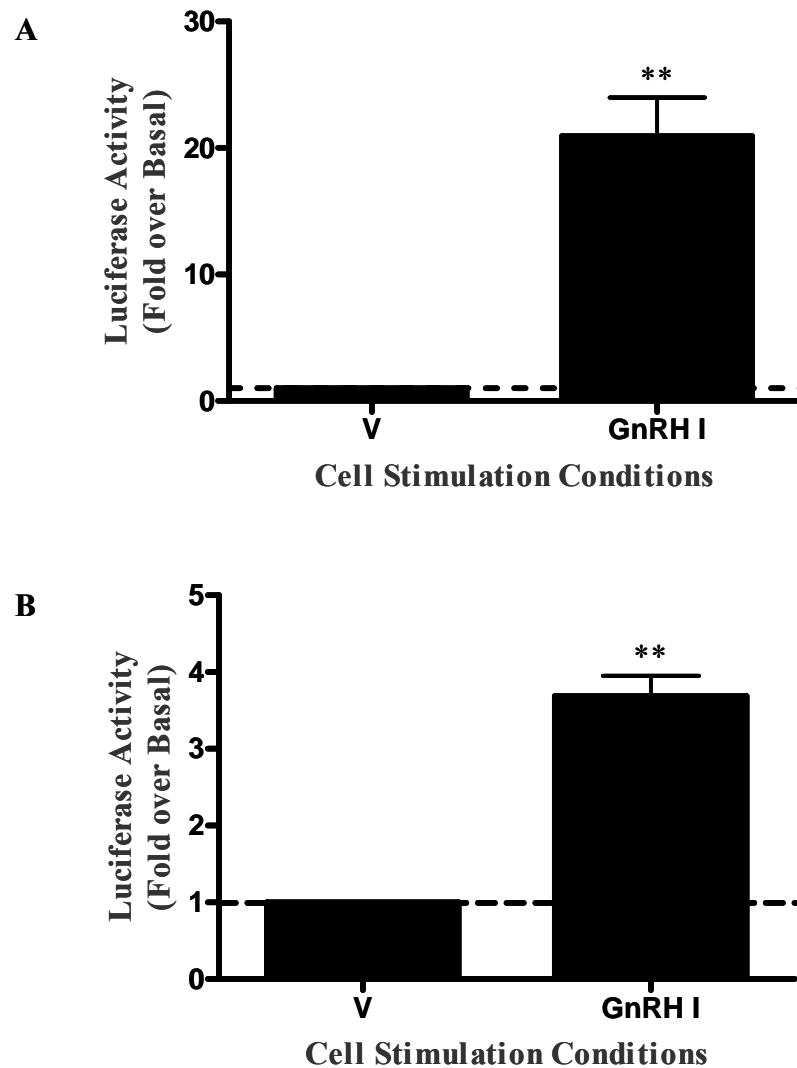


Figure 4.19 LH β - and FSH β -luciferase reporter gene assays depicting the GnRH I-induced stimulation of LH β - and FSH β -promoted transcriptional activity. L β T2 cells, stably expressing the GnRH receptor, transiently transfected with a LH β - (A) or a FSH β - (B) luciferase reporter were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I as indicated for 6 hours (A) or 8 hours (B) in the presence (B) or absence (A) of 25 ng/ml activin A. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of LH β - /FSH β -luciferase is presented. $p < 0.005$ (**) represents statistical significance from vehicle treated controls.

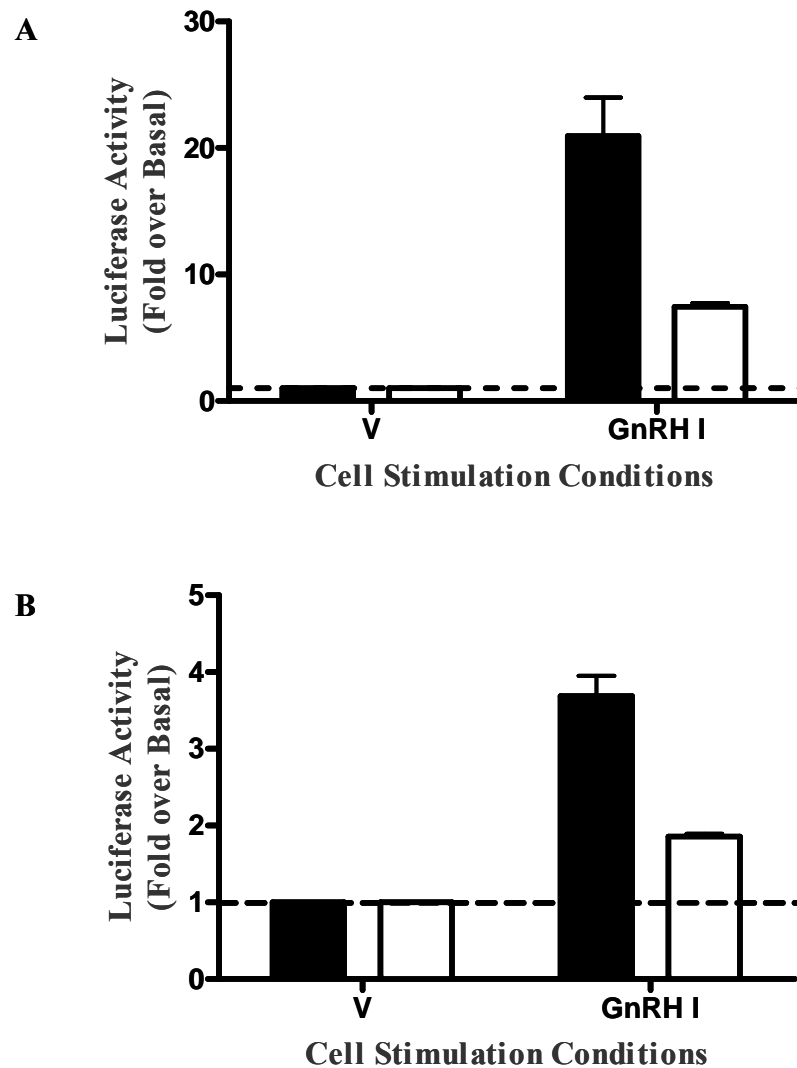


Figure 4.20 LH β - and FSH β -luciferase reporter gene assays depicting the U-73122 sensitivity of the GnRH I-induced stimulation of LH β - and FSH β -promoted transcriptional activity. L β T2 cells, stably expressing the GnRH receptor, transiently transfected with a LH β - (A) or a FSH β - (B) luciferase reporter were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 10 μ M U-73122 prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I as indicated for 6 hours (A) or 8 hours (B) in the presence (B) or absence (A) of 25 ng/ml activin A. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of LH β -/FSH β -luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.4.

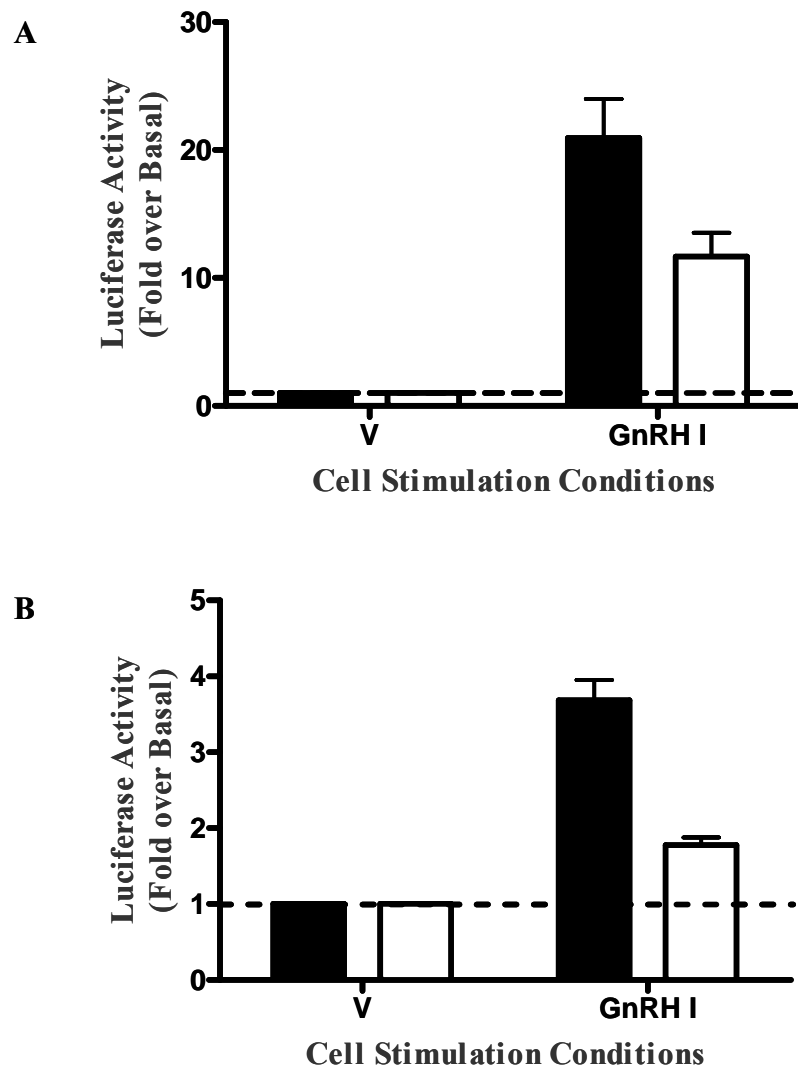


Figure 4.21 LH β - and FSH β -luciferase reporter gene assays depicting the Δ DH-p115 RhoGEF sensitivity of the GnRH I-induced stimulation of LH β - and FSH β -promoted transcriptional activity. L β T2 cells, stably expressing the GnRH receptor, transiently transfected with a LH β - (A) or a FSH β - (B) luciferase reporter and vector (black bars) or Δ DH-p115 RhoGEF (white bars) cDNA were serum starved for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I as indicated for 6 hours (A) or 8 hours (B) in the presence (B) or absence (A) of 25 ng/ml activin A. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of LH β -/FSH β -luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.4.

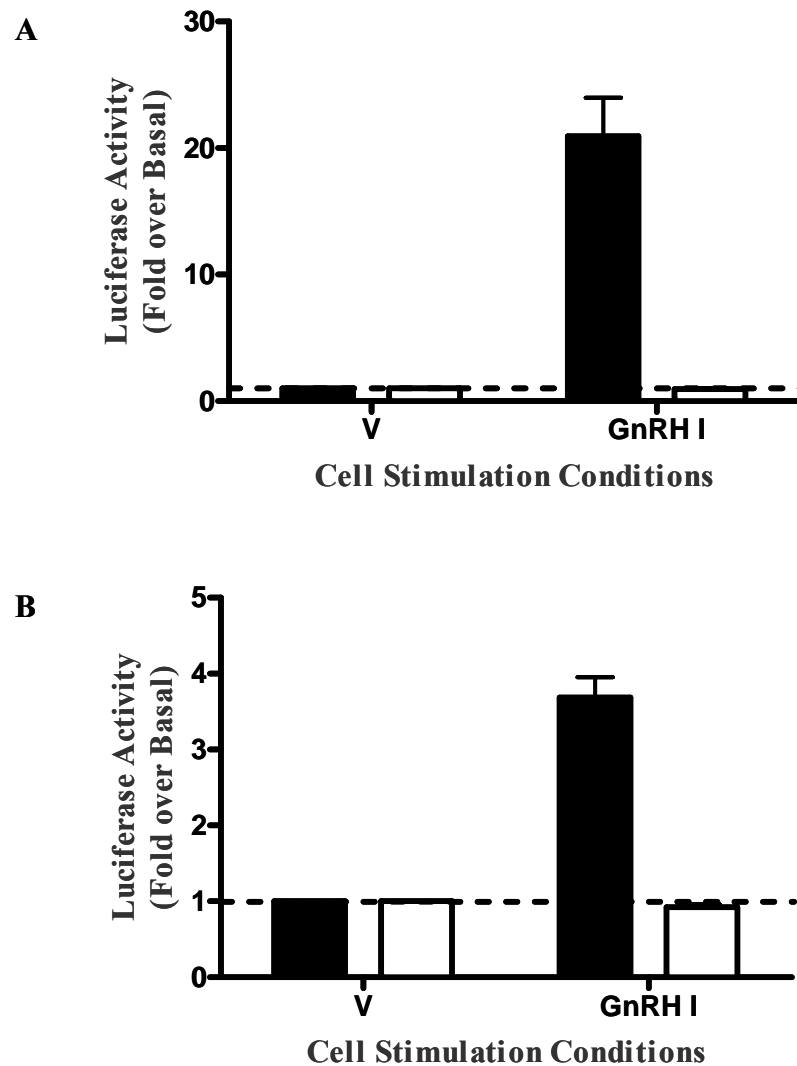


Figure 4.22 LH β - and FSH β -luciferase reporter gene assays depicting the U-73122 and Δ DH-p115 RhoGEF sensitivity of the GnRH I-induced stimulation of LH β - and FSH β -promoted transcriptional activity. L β T2 cells, stably expressing the GnRH receptor, transiently transfected with a LH β - (A) or a FSH β - (B) luciferase reporter and vector (black bars) or Δ DH-p115 RhoGEF (white bars) cDNA were serum starved in the presence (white bars) or absence (black bars) of 10 μ M U-73122 for 16 hours prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I as indicated for 6 hours (A) or 8 hours (B) in the presence (B) or absence (A) of 25 ng/ml activin A. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of LH β -/FSH β -luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.4.

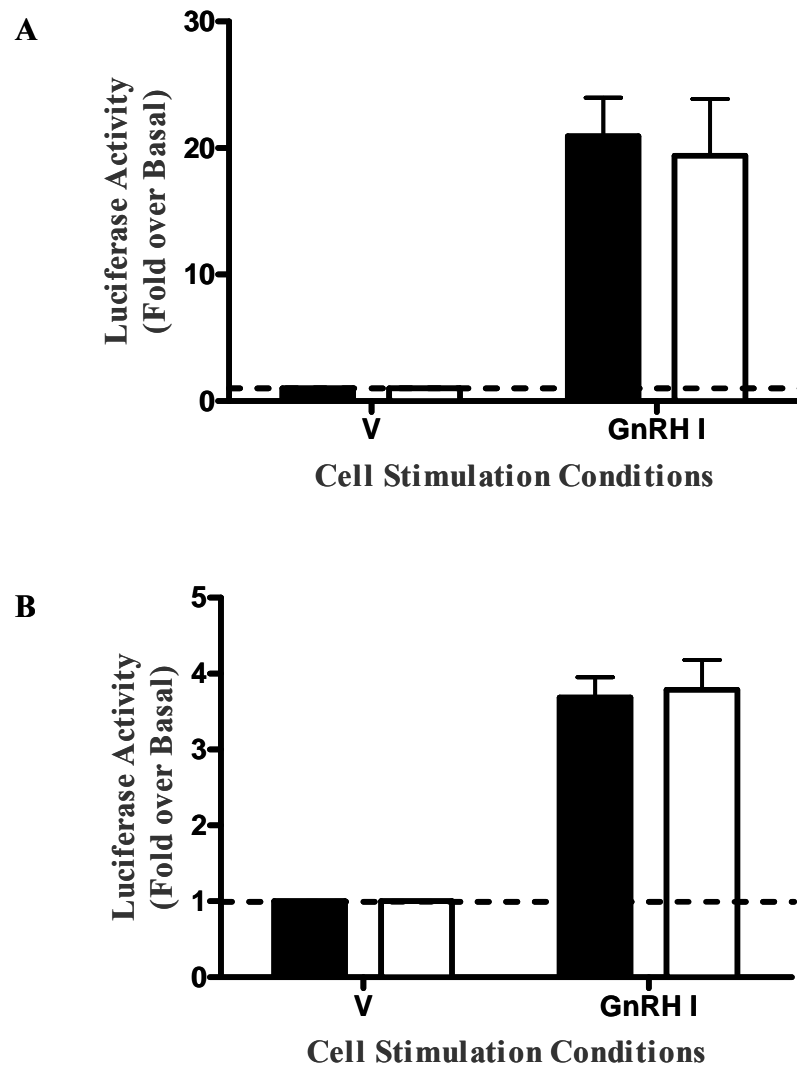


Figure 4.23 LH β - and FSH β -luciferase reporter gene assays depicting the PP2 insensitivity of the GnRH I-induced stimulation of LH β - and FSH β -promoted transcriptional activity. L β T2 cells, stably expressing the GnRH receptor, transiently transfected with a LH β - (A) or a FSH β - (B) luciferase reporter were serum starved for 16 hours in the presence (white bars) or absence (black bars) of 10 μ M PP2 prior to being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH I as indicated for 6 hours (A) or 8 hours (B) in the presence (B) or absence (A) of 25 ng/ml activin A. Luciferase activity was expressed in arbitrary units and normalised for Renilla luciferase activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of LH β -/FSH β -luciferase is presented. For ease of presentation, statistical analysis is shown in table 4.4.

Chemical Inhibitor/Dominant Negative Construct	LH β		
	Luciferase Response (Fold)	Significant Increase Above Control? [†]	Significant Inhibition? [‡]
-	20.9 \pm 3.1	**	-
U-73122	7.4 \pm 0.3	**	*
Δ DH-p115 RhoGEF	11.7 \pm 1.9	**	*
Δ DH-p115 RhoGEF + U-73122	0.9 \pm 0.1	ns	**
PP2	19.4 \pm 4.5	*	ns

Chemical Inhibitor/Dominant Negative Construct	FSH β		
	Luciferase Response (Fold)	Significant Increase Above Control? [†]	Significant Inhibition? [‡]
-	3.7 \pm 0.3	**	-
U-73122	1.9 \pm 0.0	**	**
Δ DH-p115 RhoGEF	1.8 \pm 0.1	*	**
Δ DH-p115 RhoGEF + U-73122	0.9 \pm 0.1	ns	***
PP2	3.8 \pm 0.4	*	ns

Table 4.4 Statistical analysis of LH β - and FSH β -luciferase reporter gene assays depicting the chemical inhibitor and dominant negative construct (in)sensitivity of the GnRH I-induced stimulation of LH β - and FSH β -promoted transcriptional activity. Data from at least three independent experiments were quantified and the mean fold over control \pm SE for the activation of LH β -FSH β -luciferase is presented. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) represents statistical significance from vehicle treated controls ([†]) and from GnRH treated controls ([‡]).

4.3 Discussion

Evidence suggests that the GnRH receptor may adopt multiple active conformations and thereby activate discrete intracellular signalling cascades (Lu et al., 2005). The data presented within chapter three, however, do not support the proposal that these cascades may be initiated by the GnRH-mediated activation of $G_{i/o}$ or G_s . Additionally, a large number of $G_{q/11}$ -coupled GPCRs also interact with $G_{12/13}$ (Riobo and Manning, 2005). Thus, in this chapter, the potential of GnRH to induce $G_{12/13}$ activation was investigated. The results presented herein indicate for the first time that the GnRH receptor can directly interact with the G proteins of the $G_{12/13}$ family and consequently initialise a variety of cellular responses. Additionally, the data gained during the completion of these studies confirm that the receptor may interact with src Tyr kinase and propose that GnRH I, but not GnRH II, can induce src activation. Finally, these results imply that the GnRH receptor-mediated activation of $G_{12/13}$ but not src plays an important role in the regulation of the synthesis of the gonadotropins.

Activation of the ERK cascade by GnRH has been well investigated although the precise upstream mechanism involved is controversial and has been suggested to vary between cell types (Sim et al., 1995, Naor et al., 2000, Benard et al., 2001, Liu et al., 2002a, Davidson et al., 2004, Kraus et al., 2004, Caunt et al., 2006, Maudsley et al., 2007). $G_{12/13}$ -mediated MAPK activation has also been the subject of much research (Orth et al., 2005, Honma et al., 2006, Mariggio et al., 2006, Buch et al., 2008, White et al., 2008a). Honma and colleagues recently demonstrated that the $G\alpha_{13}$ -p115 RhoGEF signalling pathway is involved in U46619-induced ERK activation in 1321N1 human astrocytoma cells (Honma et al., 2006). Similarly, Mariggio and colleagues showed that $G\alpha_{13}$ stimulates ERK phosphorylation in response to both thrombin and ATP (Mariggio et al., 2006). Consistent with these data, my results suggest the involvement of the G proteins of the $G_{12/13}$ family in GnRH-induced ERK activation. Indeed, transfection of either $G\alpha_{12}$ or $G\alpha_{13}$ into $G\alpha_{q/11}$ knockout MEF cells was sufficient to elicit an increase in ERK phosphorylation in response to GnRH stimulation. For a number of reasons, the observation that such an increase was not evident in vector transfected controls does

not preclude the above interpretation. For example, the sensitivity of the western detection system may not be high enough to detect GnRH-induced $G_{q/11}$ -independent ERK activation in this cell line. Additionally, the cellular abundance of $G_{12/13}$ may be relatively low. As such, although this assay by itself does not constitute definitive proof of GnRH receptor- $G_{12/13}$ interaction, it seems plausible to speculate that GnRH may induce $G_{12/13}$ activation. Interestingly, Liu and colleagues showed that, in L β T2 cells, GnRH elicited robust increases in ERK phosphorylation (Liu et al., 2002a). While this response could be significantly diminished by the inhibition of PKC, significant ERK activation after GnRH stimulation was still detectable. Furthermore, either the chelation of intracellular Ca^{2+} or the blockade of Ca^{2+} influx had only a very minor effect. Thus, these data support the involvement of $G_{q/11}$ -independent signalling pathways in the GnRH stimulated activation of ERK.

One of the classical responses induced by the activation of either $G\alpha_{12}$ or $G\alpha_{13}$ is the stimulation of SRE-driven transcription. In agreement with Mao and colleagues (Mao et al., 1998a), I showed that constitutively active $G\alpha_{q/11}$, $G\alpha_{12}$ and $G\alpha_{13}$, but not $G\alpha_{i/o}$ or $G\alpha_s$, could induce a SRE-luciferase response. As such, an observed increase in the luciferase signal in response to GnRH stimulation may be as a result of the GnRH receptor-mediated activation of $G\alpha_{q/11}$, $G\alpha_{12}$ or $G\alpha_{13}$. As expected, specific inhibition of $G\alpha_{q/11}$ with YM-254890 markedly decreased the SRE-luciferase response obtained after stimulation with either GnRH ligand. However, in agreement with the PMT-mediated data obtained by Orth and colleagues (Orth et al., 2005), complete inhibition of the luciferase signal by YM-254890 was not evident. I and others have shown that this compound, when used at a 100 nM concentration, completely prevents $G\alpha_{q/11}$ -induced signalling (Orth et al., 2005). These data therefore suggest the involvement of $G_{q/11}$ -independent pathways in the GnRH-induced stimulation of SRE-luciferase. Specific inhibition of the $G\alpha_{13}$ -mediated cascades with $G\alpha_{13}G225A$ or $\Delta DH-p115$ RhoGEF was sufficient to decrease the luciferase response provoked by both GnRH I and GnRH II. Significantly, p115 RhoGEF has been demonstrated to be involved in only the $G\alpha_{13}$ -Rho-induced stimulation of SRE-luciferase activity (Mao et al., 1998b). Furthermore, an expression vector carrying only the RGS domain of this protein has been shown to

inhibit the SRE-luciferase response induced by $G\alpha_{13}Q226L$ but not that mediated by $G\alpha_qQ209L$ (Chikumi et al., 2002). These results therefore suggest that the GnRH-induced SRE-driven transcription at least in part relies on the activity of the $G_{12/13}$ G protein α subunits. The observation that PTX had no effect on GnRH-induced SRE-promoted transcription was predictable for two reasons. Firstly, the data within chapter three indicate that the GnRH receptor does not couple to $G_{i/o}$. Additionally, my results, in keeping with previous observations, reveal that $G\alpha_{i/o}$ is not capable of inducing SRE-driven transcription (Fromm et al., 1997, Mao et al., 1998a). Interestingly, recent evidence suggests that the signalling cascades induced by the activation of $G\alpha_z$, a member of the $G_{i/o}$ family of G proteins, attenuate the Rho-mediated stimulation of SRE-luciferase (Dutt et al., 2004).

The $G\alpha_{13}G225A$ -mediated inhibition of the GnRH-induced stimulation of PhI hydrolysis suggests that this dominant negative mutant $G\alpha$ subunit binds tightly to the GnRH receptor thereby competitively inhibiting its interaction with $G_{q/11}$. Indeed, the observation that $G\alpha_{12}G203T$ was unable to provoke any such inhibition supports the proposal that the interaction between the GnRH receptor and $G\alpha_{13}G225A$ is direct. These data therefore indicate that, in addition to $G_{q/11}$, the GnRH receptor can directly interact with $G_{12/13}$. Additional support for this conclusion exists in the observation that transient transfection of this dominant negative G protein α subunit into COS7 cells allowed an increased amount of $G\alpha_{13}$ to be coimmunoprecipitated with the HA tagged GnRH receptor. Furthermore, even without $G\alpha_{13}G225A$ transfection, $G\alpha_{13}$ and the GnRH receptor could be coimmunoprecipitated. Analogous to these findings, coimmunoprecipitation experiments in HEK293 cells heterologously expressing the D_5 dopamine receptor revealed that both $G\alpha_{12}$ and $G\alpha_{13}$ directly interact with this GPCR (Zheng et al., 2003). Similarly, $G\alpha_s$ could be coimmunoprecipitated with the β_2 -adrenergic receptor (Lachance et al., 1999) and the angiotensin AT_2 receptor (Feng et al., 2002). My data therefore extend the number of GPCRs known to directly interact with $G_{12/13}$.

Perhaps the most intriguing finding from the results described within this chapter was the observation that GnRH I and GnRH II were capable of inducing distinct $G_{q/11}$ -independent changes in cellular morphology. Cytoskeletal reorganisation in response to the activation of the $G_{12/13}$ G proteins has been well described (Buhl et al., 1995, Fromm et al., 1997, Gohla et al., 1999, Nielsen et al., 2005, Honma et al., 2006, Wang et al., 2006, Yanagida et al., 2007). The members of this family are known to act as potent stimulators of the Rho monomeric G proteins and in doing so mediate the formation of intracellular stress fibres (Buhl et al., 1995). Furthermore, evidence suggests that ROCK plays a key role in the induction of this phenotypic response (LePage et al., 2003). The observation that GnRH II induced pronounced stress fibre formation in $G_{q/11}$ knockout cells therefore suggests, in agreement with the other data presented within this chapter, that this ligand induces $G_{12/13}$ activation and consequently stimulates Rho-ROCK signalling. This conclusion is strengthened by the observation that Y-27632 completely inhibited the GnRH II-mediated response. However, GnRH I induced the formation of a cellular morphology indicative of the activation of Rac (Ridley et al., 1992). Published evidence proposes that the $G_{12/13}$ G protein family is not involved in the positive regulation of Rac (Gratacap et al., 2001) and may even negatively regulate the activity of this monomeric G protein. Sugimoto and colleagues demonstrated that the specific inhibition of either $G_{\alpha_{12}}$ or $G_{\alpha_{13}}$ with respective carboxy-terminal peptides abolishes the sphingosine-1-phosphate- (S1P) induced $S1P_2$ -mediated inhibition of Rac (Sugimoto et al., 2003). Similarly, Yamaguchi and colleagues showed that expression of $G_{\alpha_{12}Q231L}$ in PC12 cells significantly inhibits nerve growth factor-induced Rac activation (Yamaguchi et al., 2001). Significantly, in the latter study mentioned, Y-27632 completely abolished the Rho-mediated effect. Consistent with these data, Y-27632 application resulted in the potentiation of the GnRH I-induced response thereby suggesting an increase in Rac activity. These results therefore imply that GnRH I has the ability to stimulate the activity of Rac in a $G_{q/11}$ -independent manner and that this pathway may be inhibited by ROCK. This suggestion is consistent with a previous study in which inhibition of ROCK with Y-27632 was shown to potentiate the S1P-induced src-mediated activation of Rac (Vouret-Craviari et al., 2002). GnRH II, in contrast, appears to have no such ability

to activate Rac as Y-27632 only served to completely inhibit the response obtained after stimulation.

The observation that GnRH I could induce cytoskeletal changes indicative of the activation of Rac begged the question of how this monomeric G protein was stimulated. $G_{q/11}$ -coupled GPCRs have previously been reported to be central mediators of the stimulation of Rac activity (Gratacap et al., 2001). Given that my data indicate that the GnRH I-induced stimulation of Rac occurs independently of $G_{q/11}$, I hypothesised that src may be involved in the regulation of this monomeric G protein. In support of this proposal, Davidson and colleagues showed that GnRH I-induced cytoskeletal remodelling was dependent on the activation of both src and Rac (Davidson et al., 2004) and a wealth of evidence indicates that this Tyr kinase both positively regulates Rac and negatively regulates Rho (Brandt et al., 2002, Vouret-Craviari et al., 2002, Servitja et al., 2003, Kawakatsu et al., 2005, Kai et al., 2007, Zhou et al., 2007, Dise et al., 2008). Inhibition of src with PP2 altered only the cytoskeletal reorganisation induced by stimulation with GnRH I. Specifically, PP2 application resulted in the GnRH I-mediated formation of intracellular stress fibres. These data therefore have four related implications. Firstly, they indicate that src is involved in the stimulation of Rac activity in response to GnRH I; inhibition of src completely inhibited the cellular phenotypic change indicative of Rac activation. Secondly, they suggest that, in addition to inducing the activation of Rac, GnRH I may activate the $G_{12/13}$ -Rho-ROCK-mediated pathway. Support for this interpretation comes from the fact that, upon inhibition of the src-mediated activation of Rac, GnRH I may still induce $G_{12/13}$ -Rho-ROCK signalling. Consequently, morphological changes indicative of such activation become apparent. Thirdly, they support the suggestion that GnRH II may not activate Rac; inhibition of src had no apparent effect on the cellular phenotype obtained in response to stimulation with this ligand. Finally, they imply that, in agreement with the later data presented within this chapter which will shortly be discussed, the lack of such an activation ability is due to the inability of GnRH II to, independently of $G_{q/11}$, activate src.

Src has been shown to associate directly with the intracellular domains of certain GPCRs. The β_3 -adrenergic receptor is one such example. Cao and colleagues demonstrated that ligand binding to this receptor induces the formation of a complex between src and the GPCR and that this complex can be coimmunoprecipitated in the absence or presence of arrestins (Cao et al., 2000). Protein interaction studies revealed that the receptor interacts with the SH3 domain of src via PxxP motifs in ICL3 and the carboxy-terminal tail. Similarly, Liu and colleagues showed that the P2Y₂ receptor binds the src SH3 domain via PxxP motifs in the carboxy-terminal tail (Liu et al., 2004). Recent studies, however, suggest that the SH3 domain containing proteins may bind peptide sequences that lack this canonical motif. In particular, experimental evidence has demonstrated that the (R/K)xx(K/R) sequence may instead be necessary for SH3 domain interaction (Liu et al., 2003, Jia et al., 2005). Importantly, the GnRH receptor possesses three (R/K)xx(K/R) motifs in ICL1 (figure 1.10). Thus, it is possible that, upon GnRH I-mediated receptor activation, the SH3 domain of src associates with the putative SH3 binding sites of this GPCR. Indeed, coimmunoprecipitation of src and the HA tagged receptor protein suggests, in agreement with previous research from our laboratory (Coetsee and Lu, unpublished observations), the formation of a tight complex between these proteins.

In 1998, Levi and colleagues demonstrated that GnRH increases src activity in α T31 cells (Levi et al., 1998). This increase was shown to be only partially sensitive to PKC inhibition, results which imply that src activation in part relies on signalling initiated by G_{q/11}. Later, Davidson and colleagues performed similar experiments in SCL60 cells (Davidson et al., 2004). GnRH I-induced src phosphorylation was shown to occur independently of PLC β , PKC and intracellular Ca²⁺ release. These data therefore indicate that, while there appears to be a G_{q/11}-dependent component of GnRH-mediated src activation, such signalling is not the exclusive determinant. The data described within this chapter support this concept. Indeed, significant Tyr phosphorylation of src in response to GnRH I stimulation was still detectable after complete inhibition of the G $\alpha_{q/11}$ -mediated signalling pathway with YM-254890. In contrast, and in agreement with the data described so far, no such phosphorylation was evident after stimulation with GnRH II. The studies in the MEF cells provide

additional support for this conclusion. Although not statistically significant, these data suggest that, in the complete absence of $G_{q/11}$, only GnRH I may Tyr phosphorylate src.

LiSS at the GnRH receptor is further supported by the observation that GnRH I and GnRH II induced different maximal levels of SRE-driven transcription. As with Rho, Rac is known to be a central mediator of SRE transcriptional activity (Hill et al., 1995). Indeed, transfection of a dominant negative Rac mutant into rat2 fibroblasts was sufficient to significantly inhibit $TNF\alpha$ -induced SRE-luciferase activation (Kim et al., 1999). Similarly, Rac has been shown to be necessary for the full induction of the EGF-mediated SRE-luciferase response (Kim and Kim, 1997) and EGF is known to activate Rac through a pathway involving src (Dise et al., 2008). My data also indicate that PP2 had a significant effect on the GnRH I- but not the GnRH II-induced SRE response. Thus, viewed collectively with the observation that src is involved in the activation of Rac, these findings provide evidence to support my proposal that only GnRH I has, independently of $G_{q/11}$, the ability to activate src. Consistent with my results, PP2 has been shown to impede SRE-driven transcription in PC12 cells in response to both norepinephrine and nerve growth factor (Minneman et al., 2000). Interestingly, the latter of these ligands has been shown to potently induce Rac activation in the same cell type (Yamaguchi et al., 2001).

According to the conclusions I have drawn, specific inhibition of both the $G_{12/13}$ - and the src-mediated pathways should only allow signalling induced by either ligand to be promulgated through $G_{q/11}$. At the $G_{q/11}$ activated signalling cascade, both GnRH ligands have been shown to induce PhI responses with comparable E_{max} values (Lu et al., 2007). Similarly, my work has shown that transfection of $\Delta DH-p115$ RhoGEF and subsequent application of PP2 was sufficient to induce luciferase responses of similar values after stimulation with either GnRH ligand. By themselves, these data indicate that either the $G_{12/13}$ or the src activated signalling mechanism is responsible for mediating the larger GnRH I-induced SRE-luciferase response. However, given that specific inhibition of the $G_{12/13}$ pathway had a significant effect on the SRE-

driven transcriptional output induced by both the GnRH ligands, taken together with the fact that PP2 only significantly affected such signalling induced by GnRH I, I propose that these data provide further evidence to support my interpretations that only GnRH I activates src and thus the downstream signalling cascades associated with this Tyr kinase in a $G_{q/11}$ -independent manner. Additionally, inhibition of both the $G_{q/11}$ and the $G_{12/13}$ activated cascades completely inhibited the GnRH II-induced SRE response. Such inhibition in response to GnRH I stimulation required the extra inhibition of src.

As described in chapter one, GnRH stimulated LH β and FSH β gene transcription are highly regulated processes. The results detailed within this chapter suggest that GnRH receptor- $G_{12/13}$ -mediated signalling may play a role in such regulation. While this has never been investigated, it is entirely possible for a number of reasons. With regards to LH β , Salisbury and colleagues demonstrated that maximal activity of this gene requires β -catenin (Salisbury et al., 2007) and Gardner and colleagues showed that GnRH stimulates β -catenin nuclear accumulation (Gardner et al., 2007). Interestingly, Meigs and colleagues demonstrated that constitutively active $G\alpha_{12/13}$ induces β -catenin nuclear translocation (Meigs et al., 2001). Secondly, GnRH stimulates LH β gene expression through the transcriptional regulation of EGR1 (Salisbury et al., 2007) which binds to and activates the LH β promoter by acting in combination with SF1 (Duan et al., 2002). Significantly, the 5' flanking sequence of the EGR1 gene has been cloned from several species revealing the presence of multiple SREs (Duan et al., 2002) and an EGR1 promoter may itself be activated by $G\alpha_{13}Q226L$ (Vara Prasad and Dhanasekaran, 1999). Thirdly, I and others have shown that GnRH Receptor- $G_{12/13}$ -induced signalling activates the ERK cascade (Buch et al., 2008) and ERK has shown to be involved in the GnRH-induced stimulation of LH β reporter activity (Harris et al., 2002). Finally, it has been demonstrated that $G\alpha_{13}$ endogenously expressed in HeLa cells can be coimmunoprecipitated with Pro rich Tyr kinase 2 (Pyk2) in an activation-dependent manner and that $G\alpha_{13}Q226L$ has the ability to stimulate Pyk2 kinase activity (Shi et al., 2000). Significantly, Maudsley and colleagues identified a novel GnRH

activated Pyk2-dependent signal transduction pathway that regulates EGR1 activation of the LH β promoter (Maudsley et al., 2007).

In the case of FSH β , the situation is more complex (due primarily to our lack of understanding of the molecular mechanisms underlying the transcriptional activity of this gene). Perhaps ERK may play a role in the possible G_{12/13}-mediated transcriptional regulation as this MAPK is known to be involved in the GnRH-mediated induction of FSH β synthesis (Bonfil et al., 2004). Additionally, my data, in agreement with previous findings (Orth et al., 2005), indicate that GnRH-G_{12/13}-induced signalling may activate ERK.

The involvement of src in the transcriptional regulation of LH β and FSH β is controversial. Maudsley and colleagues showed that PP2 inhibits the GnRH-induced LH β luciferase response (Maudsley et al., 2007). Clearly, these data are at odds with my observations and this discrepancy may be due to a number of reasons. For example, in the study mentioned the authors did not make use of serum deprivation prior to GnRH stimulation (Maudsley et al., 2007). Thus, GnRH-independent stimulation of LH β , which may involve src, could account for our dissimilar data. Interestingly, a recent study by Roby and colleagues demonstrated that serum LH and FSH in day 40 and day 52 mice does not differ between wild-type and src knockout animals (Roby et al., 2005).

Taken together, the results within this chapter constitute the first successful observations of direct interaction between the GnRH receptor and the G_{12/13} G proteins. Additionally, they confirm that the receptor may directly interact with src Tyr kinase and suggest that GnRH I but not GnRH II may mediate src Tyr phosphorylation and thus activation. Using a range of cellular backgrounds, I have shown that the coupling of the receptor to these discrete signalling pathways may induce a variety of cellular responses including MAPK activation, gene transcription and cytoskeletal reorganisation. These studies therefore provide a basis for further investigation into GnRH receptor-mediated ligand-induced selective signalling.

Chapter Five

Final Discussion

5.0 Final Discussion

GnRH is the central regulator of the mammalian reproductive system in that it governs the biosynthesis and the release of LH and FSH (Cheng and Leung, 2005, Pawson and McNeilly, 2005). Several studies have demonstrated that it may also inhibit cellular growth and upregulate proapoptotic signalling mechanisms (Klijn and de Jong, 1982, Dondi et al., 1994, Yano et al., 1992, Yano et al., 1994, Jeyarajah et al., 1996, Dondi et al., 1998, Kimura et al., 1999, Kraus et al., 2004, Maudsley et al., 2004, Gnanapragasam et al., 2005, Morgan et al., 2008, White et al., 2008a, White et al., 2008b). The latter action of GnRH has frequently been proposed to be mediated by the coupling of the GnRH receptor to G proteins of the $G_{i/o}$ family (Imai et al., 1996, Kimura et al., 1999, Limonta et al., 1999, Maudsley et al., 2004). Additionally, this GPCR has been proposed to couple to both $G_{q/11}$ and G_s (Liu et al., 2002b) or exclusively to $G_{q/11}$ (Grosse et al., 2000). These data therefore serve to highlight the unresolved nature of the ability of the GnRH receptor to interact with multiple G proteins. This thesis has addressed this issue in detail using, predominantly, a cell line derived from $G\alpha_{q/11}$ knockout mice (Offermanns et al., 1998). The use of this cell model may therefore promote the identification of GnRH-induced $G_{q/11}$ -independent signalling pathways.

The data presented provides strong evidence to suggest that the GnRH receptor does not couple to the G proteins of the $G_{i/o}$ family. Indeed, the observation that, even when $G\alpha_{q15}$ was expressed, GnRH did not induce significant phosphorylation of ERK, JNK or P38 dictates that this GPCR does not interact with $G_{i/o}$. This conclusion is strengthened by the observation that $G_{q/11}$ -mediated signalling can clearly facilitate the GnRH-induced activation of all the MAPKs studied. Thus, the activation of a $G_{i/o}$ -coupled GPCR in cells expressing $G\alpha_{q15}$ should result in the phosphorylation of these proteins. My interpretations are consistent with previous studies which investigated the most proximal step in GnRH-induced G protein activation; GDP/GTP exchange (Grosse et al., 2000, White et al., 2008a). Conversely, they are not easily reconcilable with several investigations that use PTX as a method to infer GnRH receptor- $G_{i/o}$ interaction (Sim et al., 1995, Imai et al., 1996, Kimura et al., 1999, Limonta et al., 1999, Maudsley et al., 2004).

Nevertheless, as described in chapter one, while positive responses to chemical inhibitors provide useful preliminary indications of GPCR-G protein interaction, they are by themselves not sufficient to accurately interpret experimental data.

The observation that GnRH did not induce any increase in cAMP suggests that the GnRH receptor does not couple to G_s . It would seem improbable that this assay was too insensitive to detect any such increase given the 14.7 ± 0.4 -fold response elicited by isoproterenol. It is plausible to speculate that the $G\beta\gamma$ complex-mediated inhibition of adenylyl cyclase isoforms may mask any GnRH receptor- G_s -mediated cAMP increase. However, given that the $G\beta\gamma$ complex also stimulates the activity of certain adenylyl cyclase isoforms and thus increases cAMP levels (Gao and Gilman, 1991, Tang and Gilman, 1991, Yoshimura et al., 1996), this would appear unlikely. These data therefore provide evidence to indicate that GnRH receptor- G_s interaction may not occur. This conclusion is supported by a previous investigation which utilised direct analytical techniques to analyse GnRH-induced G protein activation (Grosse et al., 2000). In this study, the authors could obtain no evidence to indicate that the GnRH receptor couples to G_s . Additionally, unpublished work from our laboratory indicates that GnRH may not induce a CRE-mediated transcriptional response suggesting that, even at the most distal level, activation of G_s by the GnRH receptor is undetectable (Rischitor and Pawson, unpublished observations).

My finding that the GnRH receptor directly interacts with the $G_{12/13}$ G proteins is one that has not been widely investigated. Nevertheless, the fact that, in contrast to $G\alpha_{i2}G203T$, $G\alpha_{i3}G226A$ could competitively inhibit the GnRH-induced PhI hydrolysis suggests that the GnRH receptor may directly interact with these G protein α subunits. Coimmunoprecipitation of both endogenous $G\alpha_{i3}$ and $G\alpha_{i3}G225A$ with the HA tagged GnRH receptor provide support for this interpretation. Additionally, my results imply that coupling of the receptor to $G_{12/13}$ may be functionally important in the synthesis of the gonadotropins. A more detailed analysis is, however, required in order to elucidate the precise mechanism involved. Interestingly, Enomoto and colleagues recently revealed that GnRH induced Rho-dependent stress fibre formation in DU145 cells (Enomoto et al., 2006).

Moreover, I have shown that, in agreement with previous investigations (Buhl et al., 1995, LePage et al., 2003), GnRH-induced activation of $G_{12/13}$ regulates the formation of stress fibres in a manner dependent on the activation of Rho and consequently ROCK. Thus, perhaps the GnRH-mediated activation of $G_{12/13}$ is involved in the phenotypic change observed in the investigation highlighted. Additionally, the $G_{12/13}$ G proteins are highly implicated in cellular migration and invasion (Offermanns et al., 1997, Kelly et al., 2006a, Kelly et al., 2006b, Kelly et al., 2007, Worzfeld et al., 2008). Cheung and colleagues recently showed that low doses of GnRH stimulated Caov3 and OVCAR3 cellular motility and invasion (Cheung et al., 2006). GnRH receptor activation of the $G_{12/13}$ G proteins may, in this case, be functionally important.

One of the main aims of this thesis was to clarify the possible mechanisms underlying the ability of GnRH I to induce the inhibition of cellular growth. In this regard, a thorough elucidation of the G protein coupling ability of the GnRH receptor was necessary. My results clearly show that $G_{q/11}$ is an absolute requirement for GnRH I-induced antiproliferation. It came as a surprise, therefore, that the inhibition of PKC or the chelation of intracellular Ca^{2+} had no significant effect. Based on both my later data (which indicate that, in the presence of $G_{q/11}$, src is important in the GnRH I stimulated antiproliferative response) and previous research within our group (Coetsee and Lu, unpublished observations), I proposed that SHP-2 may be involved in this process. In this regard, Tyr phosphorylation of the GnRH receptor is likely to be necessary as the pYxxL motif is known to provide a docking site for SHPs (Unkeless and Jin, 1997). Src activation is known to result in the Tyr phosphorylation of certain GPCRs such as the OX_1 receptor (Voisin et al., 2008) and the somatostatin SST_2 receptor (Ferjoux et al., 2003). Additionally, Coetsee and colleagues have demonstrated that GnRH receptor Tyr phosphorylation is highly correlated with src association (Coetsee and Lu, unpublished observations). In the context of my data, however, the question of how src is activated in response to GnRH I stimulation still remained.

There are a number of pathways which may induce the Tyr phosphorylation and thus the activation of src. Based on the observations presented within chapter four, src activation may occur downstream of $G_{q/11}$ and, in response to GnRH I stimulation, independently of these G proteins. Src has also been shown to become activated downstream of the $G\beta\gamma$ complex (Shajahan et al., 2004, Gentili et al., 2006). With reference to the antiproliferative effects of GnRH I, it would appear that the $G\alpha_{q/11}$ -induced activation of src does not play a major role; inhibition of the $G\alpha_{q/11}$ -mediated signalling pathway had no effect on the antiproliferative response obtained. The same conclusion may be reached for the possible GnRH I-induced src activation downstream of the $G\beta\gamma$ complex. Indeed, the data in chapter four indicate that GnRH II does not, independently of $G_{q/11}$, result in src activation. However, activation of $G_{12/13}$, which can be induced by GnRH II, will result in $G\beta\gamma$ release. These data therefore suggest that the $G\beta\gamma$ complex, in response to GnRH stimulation, does not induce src activation. Coimmunoprecipitation studies revealed that src forms a tight and constitutive complex with the GnRH receptor. Taken together with the presence of three putative SH3 binding domains in ICL1, it is conceivable that src may, in response to GnRH I stimulation, bind to and become activated directly by this GPCR. Presumably, GnRH I binding facilitates receptor conformational changes such that the (R/K)xx(K/R) motifs become accessible.

Given that GnRH I stimulation can result in src activation independently of $G_{q/11}$, that src association is known to correlate with the Tyr phosphorylation of the GnRH receptor (Coetsee and Lu, unpublished observations) and that the pYxxL motif is a docking site for SHPs (Unkeless and Jin, 1997), SHP-2 may be expected to interact with this GPCR after GnRH I stimulation. Such interaction may control the duration and signalling intensity of ERK, a MAPK that I have shown is important in the GnRH I stimulated inhibition of cell growth. A key unresolved issue, however, is where $G_{q/11}$ fits into this scheme. Based on research performed within our laboratory, and similar to the data obtained by Feng and colleagues (Feng et al., 2002), I propose that $G_{q/11}$ acts more as a scaffold for the binding of SHP-2 to the GnRH receptor. In this regard, the presence but not the activation of these G proteins would be necessary. Perhaps the YxxL motif in the $G\alpha_{q/11}$ subunits plays a role in

this scaffolding complex as SHP-2 has two distinct SH2 domains and the engagement of both is known to be necessary for maximum phosphatase activity (Neel et al., 2003). In support of this suggestion, Coetsee and colleagues have demonstrated that SHP-2 activation requires the presence of $G_{q/11}$ (Coetsee and Lu, unpublished observations). Additionally, these investigators have shown that GnRH I stimulation of $G_{q/11}$ -uncoupled GnRH receptor mutants results in robust SHP-2 activation and that $G_{q/11}$ and SHP-2 may be coimmunoprecipitated (Coetsee and Lu, unpublished observations).

The proposal described above highlights a potential mechanism whereby GnRH I may induce cell growth inhibition. Nevertheless, it is difficult to reconcile with certain previous studies. For example, complete inhibition of $G_{q/11}$ -induced signalling with YM-254890 is known to completely inhibit the GnRH I-induced antiproliferative response in SCL60 cells (White et al., 2008a). Additionally, in the same cell type, the specific inhibition of PKC has been shown to have the same effect (Morgan et al., 2008) and GnRH II has been postulated to be more potent in inducing antiproliferation than GnRH I (Lopez de Maturana et al., 2008). Recent investigations have highlighted the importance of cellular context in this GnRH-induced response (Morgan et al., 2008). Thus, the data discussed previously may provide an additional pathway whereby GnRH I may inhibit cell growth. I would argue, however, that it is highly unlikely to be the only cascade involved in the regulation of such a process.

The results presented within this thesis have provided strong evidence to indicate that the GnRH receptor does not couple to the G proteins of either the $G_{i/o}$ or the G_s families. In doing so, they have highlighted a crucial role for $G_{q/11}$ in the GnRH I-induced inhibition of cell growth. Additionally, I have identified a novel GnRH receptor- $G_{12/13}$ signalling pathway, proposed a role for this pathway in the GnRH-mediated regulation of the gonadotropins and provided evidence to support the concept of LiSS. GnRH continues to offer the promise of extra therapeutic value. In order to exploit such potential, however, it is essential that the signalling pathways initiated downstream of receptor activation are accurately delineated. Perhaps the

studies detailed herein may help refocus the field and aid in the translation of basic research into clinical practice.

Chapter Six

References

6.0 References

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Chapter Seven

Appendices

7.0 Appendices

The information contained within these appendices is pertinent to the data presented within this thesis. The first details the abbreviations and the one letter codes of the amino acids used throughout the text and the second the publications completed or contributed to during the course of this PhD.

7.1 Amino Acid Abbreviations and One Letter Codes

The abbreviations and the one letter codes of the amino acids used throughout the text are shown in table 7.1.

Amino Acid	Abbreviation	One Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Table 7.1 Amino acid abbreviations and one letter codes.

7.2 Publications

The publications completed or contributed to during the course of this PhD are shown on the following pages. Permission has been granted by all the authors and the respective editorial offices allowing the inclusion of the manuscripts within this thesis.

Structural Determinants for Ligand-Receptor Conformational Selection in a Peptide G Protein-coupled Receptor*

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G protein coupled receptors (GPCRs) modulate the majority of physiological processes through specific intermolecular interactions with structurally diverse ligands and activation of differential intracellular signaling. A key issue yet to be resolved is how GPCRs developed selectivity and diversity of ligand binding and intracellular signaling during evolution. We have explored the structural basis of selectivity of naturally occurring gonadotropin-releasing hormones (GnRHs) from different species in the single functional human GnRH receptor. We found that the highly variable amino acids in position 8 of the naturally occurring isoforms of GnRH play a discriminating role in selecting receptor conformational states. The human GnRH receptor has a higher affinity for the cognate GnRH I but a lower affinity for GnRH II and GnRHs from other species possessing substitutions for Arg⁸. The latter were partial agonists in the human GnRH receptor. Mutation of Asn^{7,45} in transmembrane domain (TM) 7 had no effect on GnRH I affinity but specifically increased affinity for other GnRHs and converted them to full agonists. Using molecular modeling and site-directed mutagenesis, we demonstrated that the highly conserved Asn^{7,45} makes intramolecular interactions with a highly conserved Cys^{6,47} in TM 6, suggesting that disruption of this intramolecular interaction induces a receptor conformational change which allosterically alters ligand specific binding sites and changes ligand selectivity and signaling efficacy. These results reveal GnRH ligand and receptor structural elements for conformational selection, and support co-evolution of GnRH ligand and receptor conformations.

G protein-coupled receptors (GPCRs)² constitute the largest family of signaling molecules in the mammalian genome. Over

800 GPCRs have been identified in the human genome (1). GPCRs bind a variety of structurally diverse ligands ranging from photons, ions, bioamines, lipids, nucleotides, and peptides to large polypeptide hormones at the extracellular surface. They activate a number of different intracellular effector proteins including G proteins or non-G proteins which participate in the majority of physiological processes. About 50% of current clinical drugs target GPCRs, and these receptors thus remain a major avenue for future drug development.

The 7-TM GPCRs are presumed to have evolved from a common ancestor (2), and are thought to share important structural and functional characteristics (3–5), but have undergone specialization to mirror the nature of their cognate ligands. It is not clear, however, how GPCRs developed ligand selectivity to cognate ligands during evolution. We hypothesized that GPCR binding specificity is not only determined by ligand contact residues but also by receptor conformations specified by receptor intramolecular interactions. There is also increasing evidence that ligands can selectively stabilize different receptor active conformations thereby mediating ligand-induced selective signaling (LiSS) (6–9). Selection of signaling by analogues clearly has potential for future drug development with novel activities and reduced side-effects. Hence, delineation of receptor allosteric communication networks which couple selective ligand structural elements to specific receptor conformational changes is fundamental to understanding LiSS (10).

Although only one functional member of the GnRH receptor and two isoforms of GnRH ligands (GnRH I and GnRH II) (Fig. 1A) exist in humans, coexistence of multiple types of GnRH ligands and receptors was identified in the majority of chordate and vertebrate species (6). The human GnRH receptor has high affinity for GnRH I (Fig. 1, A and B) but a 10-fold lower affinity for GnRH II, which differs by three amino acids (Fig. 1A). In contrast, the marmoset and macaque type II GnRH receptors have a high affinity for GnRH II but a much lower affinity for GnRH I (11). The human type II GnRH receptor has been silenced by stop codons and frameshift deletions (6, 12), suggesting that the single subtype of the human GnRH receptor mediates actions of both ligands. The ligand binding sites identified in the human GnRH receptor for the conserved N- and C-terminal amino acids of GnRHs (Fig. 1C) are almost fully conserved among all GnRH receptors (6). This implies that the evolutionarily variable residues in position 5, 7, and 8 of the jawed vertebrate GnRHs confer ligand binding and functional selectivity (6, 13). We have recently revealed that mutations in the single subtype of the human GnRH receptor remote from

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² The abbreviations used are: GPCRs, G protein-coupled receptors; TM, transmembrane domain; GnRH, gonadotropin-releasing hormone; LiSS, ligand-induced selective signaling; PIP, phosphoinositide; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; mAChR, muscarinic acetylcholine receptor; PDB, Protein Data Bank.

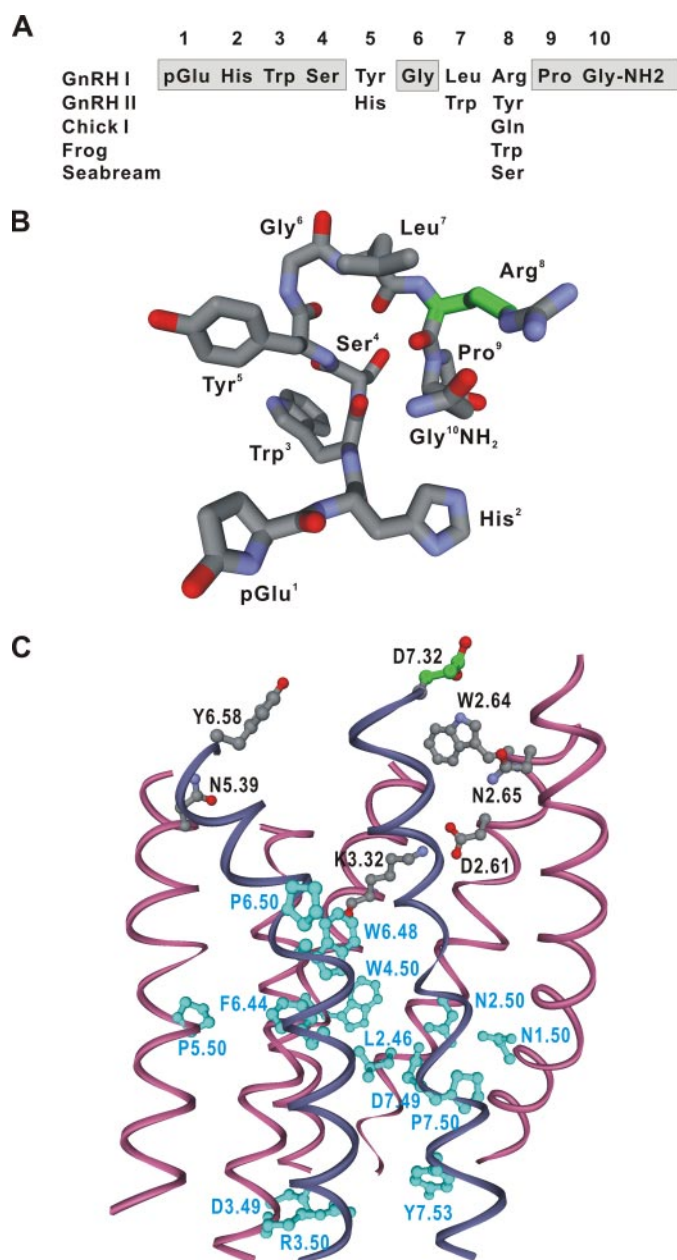


FIGURE 1. Structures of GnRHs and the human GnRH receptor. A, primary structures of GnRH I, GnRH II, and GnRHs from other species with Arg⁸ substitution. The N-terminal amino acids (pGlu¹-His²-Trp³-Ser⁴) and C-terminal amino acids (Pro⁹-Gly¹⁰-NH₂) of the decapeptide ligands are highly conserved over 600 million years of the chordate evolution and are important for ligand binding (see below) and receptor activation (6). B, an NMR structure of GnRH I (PDB code: 1YY1) showing a β II' conformation. C, a homology model of the 7-TM domains of the human GnRH receptor in the inactive state. The ligand binding residues for GnRH I are labeled in black. pGlu¹ is proposed to interact with Asn^{5.39}; His² with Asp^{2.61}/Lys^{3.32}; Tyr⁵ with Tyr^{6.58}; Arg⁸ with Asp^{7.32}; and Gly¹⁰NH₂ with Asn^{2.65}. These interactions can all be satisfactorily accommodated when GnRH in the β II' conformation is docked to the receptor (not shown for clarity) (6, 10). There is no intermolecular interaction between Tyr⁸ of GnRH II and Asp^{7.32} (green) that interacts with Arg⁸ of GnRH I (green) (41). The most highly conserved (80–100%) residues in the 7 TM domains among rhodopsin-like family of GPCRs are colored blue.

ligand binding sites have differential effects on the binding affinity of the two endogenous ligands (10), implying differential ligand-receptor conformational selections. Here we present studies supporting the hypothesis that changes in the GnRH receptor conformation occurred coincident with amino acid

changes of GnRH ligands, which modify ligand structure/conformation, *i.e.* there was a reciprocal structural/conformational selection between ligands and receptors during evolution.

Our preliminary screening mutagenesis of putative TM interacting residues revealed candidates which appeared functionally important for ligand binding and receptor activation for further studies. Here we report that the highly conserved Asn^{7.45} in TM 7 makes intramolecular interactions with a highly conserved Cys^{6.47} in TM 6 which plays an important role in control of receptor conformational states of the human GnRH receptor, involved in binding selectivity and signaling efficacy of GnRH analogues, which differ by only one amino acid in position 8.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Receptor Expression—A PCR method was used to construct mutant receptors of N7.45A, C6.47A, and C6.47Y. The mutant receptor DNAs cloned into the pcDNA I expression vector were validated by di-deoxy sequencing. Wild-type and mutant receptors were transiently expressed in COS-7 cells by transfection using a Bio-Rad Gene Pulser at 230 V and 960 microfarads with 20 μ g of DNA/0.4-cm cuvette (1×10^7 cells; 0.7 ml). After transfection, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics, and 2 mM glutamine (complete DMEM) in the absence or presence of 1 μ M IN3 (a membrane-permeant non-peptide GnRH receptor antagonist). Cells were washed four times, each wash lasted for 30 min, with 2% Me₂SO, 0.1% BSA/Hepes/DMEM at 37 °C after a 48-h incubation. The cells were then incubated with complete DMEM overnight (~18 h), and were washed again as above prior to assaying. This allowed complete removal of IN3 from pretreated cells before assaying (10).

Ligand Binding—Radioligand binding assays were performed on intact cells 72 h after transfection (10). Transfected cells in 12-well culture plates were washed as above and then incubated with ¹²⁵I-[His⁵,D-Tyr⁶]GnRH (100,000 cpm/0.5 ml/well) and various concentrations of unlabeled GnRH ligands in 0.1% BSA/Hepes/DMEM for 4 h at 4 °C. Nonspecific binding was determined in the presence of 1 μ M unlabeled GnRH I. After incubation, the cells were rapidly washed with 1 ml ice-cold phosphate-buffered saline (pH 7.4) twice and solubilized in 0.5 ml of 0.1 M NaOH. Radioactivity was counted by γ -spectrometry. All experiments were performed in triplicate and repeated at least three times.

PhI Hydrolysis—Assays for ligand stimulation of inositol phosphate production were previously described (10). Transfected COS-7 cells were seeded onto 12-well plates in the absence or presence of 1 μ M concentration of IN3. After 48 h, IN3 was washed off as above and labeled overnight with 1 μ Ci/ml myo-D-[³H]inositol in inositol-free DMEM containing 1% dialyzed fetal calf serum. Before conducting PhI assay, the cells were washed again as above. Cells were then preincubated with 0.5 ml of buffer A (140 mM NaCl, 20 mM HEPES, 8 mM glucose, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mg/ml BSA) containing 10 mM LiCl at 37 °C for 30 min, followed by addition of GnRHs for an additional 30 min. The stimulation was terminated by removal of the medium and addition 0.5 ml of 10 mM

formic acid. The [^3H]inositol phosphates were isolated from the formic acid extracts using Dowex AG 1-X8 ion exchange resin and collected with 1 M ammonium formate containing 0.1 M formic acid and quantified by liquid scintillation counting.

Comparative Modeling of the GnRH Receptor and Molecular Dynamics (MD) Simulations—Initial homology models of the human GnRH receptor in the inactive or active state were built on the crystal structures of bovine rhodopsin (14, 15) (PDB codes 1U19 and 2I37) using a similar method as previously described (10, 16, 17), with the “MODELLER” module within DS Modeling (version 1.6, Accelrys, San Diego, CA). Although there are concerns in regard to the use of the rhodopsin structure as a template to model other GPCRs due to low sequence similarities among the rhodopsin-like family of GPCRs, sequence analysis suggested that GPCRs share a similar arrangement of the 7-TM domains. This is also because of the presence of a few, but significantly conserved residues and motifs in each of the 7-TM domains (4, 18, 19). The amino acids possessing 80–100% conservation are Asn^{1.50}, Leu^{2.46}, Asp/Asn^{2.50}, Cys^{3.25}, Glu/Asp^{3.49}, Arg^{3.50}, Trp^{4.50}, Phe^{6.44}, Trp/Phe^{6.48}, Pro^{6.50}, Pro^{7.50}, and Tyr^{7.53} (4, 18) (Fig. 1C). Molecular modeling of GPCRs has recently been extensively reviewed by Fanelli and De Benedetti (18) in which they suggest that comparative modeling of the 7-TM bundle of GPCRs using rhodopsin structure as a template is likely to produce reliable results. The use of rhodopsin to comparatively model the mammalian GnRH receptor has been validated by the authors (6, 10) and also by independent groups using extensive site-directed mutagenesis studies and peptide (20, 21) and non-peptide docking supported by 76 mutations (22). The MODELLER-generated models with the highest values of the three-dimensional profile score, computed by means of the module of “verify protein” in the DS modeling, were selected for further refinement. The models incorporating all previously experimentally identified amino acid interactions were subjected to *in vacuo* energy minimization and MD simulations by means of the CHARMM program (23), using a setup similar to that described for the modeling of the closely-related oxytocin receptor (24). Harmonic restraints of 2.5 kcal/mol/Å² on the receptor backbone atoms except for the second extracellular loop and the experimentally identified disulfide-bonded N-terminal domain (25) were applied to allow small conformational changes of the receptor during the MD simulations without loss of the overall receptor topology (26). Minimizations were carried out by using 1500 steps of steepest descent followed by a conjugate gradient minimization, until the root-mean-square gradients was less than 0.001 kcal/mol/Å. A distance-dependent dielectric term ($\epsilon = 4r$) and a 12 Å non-bonded cut-off distance were chosen. The system was heated to 300 K with 5 K rise, every 100 steps per 6000 steps, by randomly assigning velocities from the Gaussian distribution. After heating, the system was allowed to equilibrate for 34 ps. Finally, a production phase was carried out involving a 100 ps simulation using an NVT ensemble at 300 K, with a time steps of 1 fs. The models were minimized as above and used for the comparative analysis.

Materials—GnRH I and GnRH II were purchased from Sigma and Bachem. [His⁵,D-Tyr⁶]GnRH, [His⁵]GnRH, [Trp⁷]GnRH, [Tyr⁸]GnRH, chicken GnRH I ([Gln⁸]GnRH),

frog GnRH ([Trp⁸]GnRH), and seabream GnRH ([Ser⁸]GnRH) were synthesized in our laboratory as previously described (10). IN3, (2*S*)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxoethyl]-2-(3,5-dimethylphenyl)-1*H*-indol-3-yl]-*N*-(2-pyridin-4-ylethyl) propan-1-amine was obtained from MERCK (10).

Data Analysis—Binding curves were fitted to the Hill equation or to the one-site model using Sigmaplot 9.0 (SPSS) yielding an IC₅₀ value. The receptor expression levels were calculated as percentage of the wild-type control included in each transfection. PhI dose-response curves were fitted to a four-parameter logistic function, yielding a basal activity, a maximum response (E_{max}), an EC₅₀ value and a slope factor.

RESULTS

Mutation of Asn^{7.45} to Ala Induces a Receptor Conformational Instability That Is Rescued by a Membrane-permeant Non-peptide GnRH Antagonist—Mutation of Asn^{7.45} to Ala completely abolished receptor expression on cell surfaces when transiently transfected into COS-7 cells, as measured by ligand binding assays with a hydrophilic peptide agonist [¹²⁵I]-[His⁵,D-Tyr⁶]GnRH on intact cells (Fig. 2A, *inset*). The mutant receptor also gave undetectable PhI responses.³ To investigate if a cell membrane-permeant non-peptide GnRH antagonist, IN3, could rescue the mutant receptor by chaperoning it to the cell surface, the wild-type and mutant receptor transfected COS-7 cells were preincubated with 1 μM IN3 for 48 h. After washing out the IN3 as described previously (10), the expression level of the mutant receptor was measured by radioligand binding giving 40% of the wild-type level (Fig. 2A, *inset*). The action of the chaperone ligand IN3 on receptor expression was observed not only in the mutant GnRH receptor but also in the wild-type receptor (Fig. 2A, *inset*). Pretreatment of the receptor-transfected COS-7 cells with IN3 had no effect on GnRH ligand binding affinity in the wild-type receptor after washout (Fig. 2A). This result suggests that the membrane-permeant non-peptide GnRH antagonist IN3 can bind with the newly synthesized receptor inside of cells, and alter receptor conformations from an unstable to a more stable state.

Effect of Mutation of Asn^{7.45} to Ala on Receptor Binding Affinity of GnRH Analogues—The mutation N7.45A had little effect on binding affinity of GnRH I (Fig. 2B) and [His⁵,D-Tyr⁶]GnRH, which we conventionally use as a radioligand (Fig. 2A), but increased affinity of the mutant receptor for GnRH II by 8-fold, as compared with the affinity of the wild-type receptor for GnRH II (Fig. 2B and Table 1). There are three amino acid differences between GnRH I and GnRH II in which Tyr⁵, Leu⁷, and Arg⁸ of GnRH I are replaced by His⁵, Trp⁷, and Tyr⁸ in GnRH II (Fig. 1A). We examined the effect of mutation of Asn^{7.45} to Ala on the binding affinity of GnRH I analogues with single amino acid substitutions (His⁵, Trp⁷, or Tyr⁸). The mutation N7.45A had no significant effect on the receptor binding affinity for [His⁵]GnRH and [Trp⁷]GnRH, but increased receptor binding affinity for [Tyr⁸]GnRH by 14-fold (Fig. 2C and Table 1).

³ Z. L. Lu, unpublished observation.

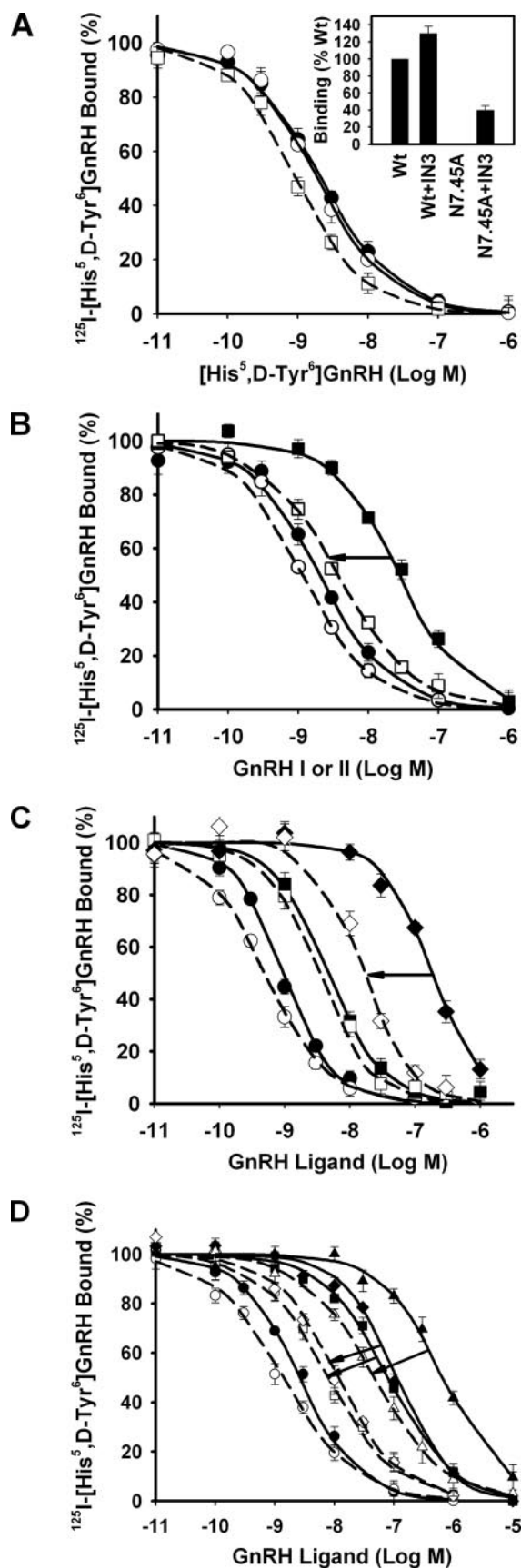


FIGURE 2. Competitive binding of GnRH analogues at wild-type and N7.45A mutant receptors. The wild-type and N7.45A mutant receptor transfected COS-7 cells were preincubated with or without 1 μM IN3 for 48 h. The

The binding of GnRH I, which contains Arg⁸, to the wild-type human GnRH receptor was characterized by one-site binding isotherms ($n_H = 0.86$) with an IC_{50} at 2.6 nM. The binding affinities of the wild-type human GnRH receptor for GnRHs from other species which only have one amino acid difference in position 8, including chicken GnRH I ([Gln⁸]GnRH), frog GnRH ([Trp⁸]GnRH), and seabream GnRH ([Ser⁸]GnRH) (Fig. 1A) were much lower than GnRH I with IC_{50} values at 80 nM, 111 nM, and 684 nM (Fig. 2D and Table 1). Interestingly, mutation of Asn^{7.45} to Ala also increased the mutant receptor affinity for chicken GnRH I ([Gln⁸]GnRH), frog GnRH ([Trp⁸]GnRH), and seabream GnRH ([Ser⁸]GnRH) by 10-fold as was seen for [Trp⁸]GnRH (Fig. 2D and Table 1).

Effect of Mutation of Asn^{7.45} to Ala on the GnRH Ligand-elicited PhI Turnover—When the N7.45A mutant receptor expression was rescued, GnRHs were able to elicit a functional response. Interestingly, GnRH I and GnRH II elicited a maximum phosphoinositide (PhI) response in the N7.45A mutant receptor of 110–122% that of the wild-type receptor (Fig. 3A) even though its expression was only 40% of the wild-type level, suggesting increased signaling efficacy. In parallel with the increased GnRH II binding affinity, the mutant receptor was also more potent in eliciting PhI response, leading to a 3-fold decrease in the EC_{50} value as compared with the wild-type receptor (Fig. 3A and Table 2).

Activation of GnRH receptors from different species by their cognate ligands can selectively couple to different members of the G protein family such as $G_{q/11}$, G_s , and $G_{i/o}$. The human GnRH receptor preferentially couples to $G_{q/11}$, although coupling to G_s and $G_{i/o}$ was reported in certain cell types (6). GnRH I elicits a robust PhI response from COS-7 cells transfected with the human GnRH receptor, giving a maximum response five times the basal activity and an EC_{50} value of 0.2 nM (Fig. 3, A and B). Chicken GnRH I ([Gln⁸]GnRH), frog GnRH I ([Trp⁸]GnRH), and seabream GnRH I ([Ser⁸]GnRH) were able to activate $G_{q/11}$ -mediated PhI turnover in the human GnRH receptor with increased EC_{50} values (Table 2). However, all of them acted as partial agonists giving reduced E_{max} responses at 44–83% of that elicited by GnRH I (Fig. 3B and Table 2). Most interestingly, all of them became full agonists in the N7.45A mutant receptor, even though the expression is only 40% of the wild-type, yielding E_{max} responses equal to or greater than that elicited by GnRH I in the wild-type receptor with little or only a small effect on the signaling potency (EC_{50} value) (Fig. 3B and Table 2).

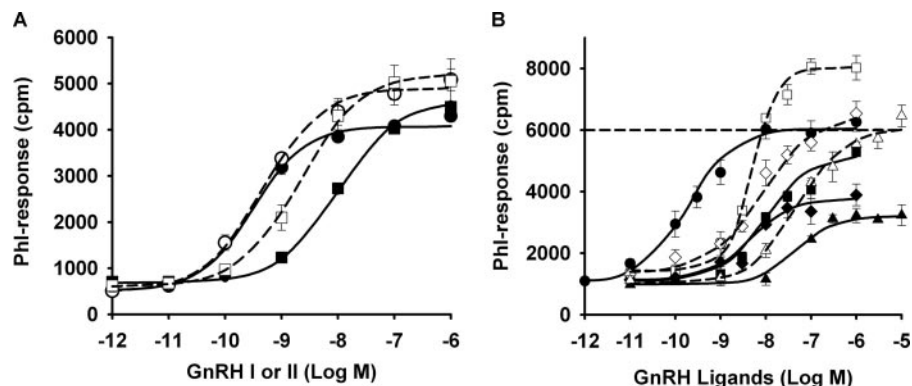
IN3 was washed off prior to binding assays. A, homologous binding of [His⁵,D-Tyr⁶]GnRH, mutation of Asn^{7.45} to Ala led to an undetectable GnRH ligand binding, which was rescued by IN3 preincubation of the transfected cells (inset). There was no difference in the GnRH binding affinity between the IN3 pretreated and the untreated cells of wild-type receptors. ●, wild-type; ○, wild-type with IN3 pretreatment; □, N7.45A with IN3 pretreatment. B, competitive binding of GnRH I (● and ○) and GnRH II (■ and □) at the wild-type and N7.45A mutant receptors. C, competitive binding of GnRH I analogues with single amino acid substitution of GnRH II in GnRH I at position 5, 7, or 8 at the wild-type and N7.45A mutant receptors, [His⁵]GnRH (● and ○); [Trp⁷]GnRH (■ and □); [Trp⁸]GnRH (◆ and ◇). D, binding of GnRHs from different species with Arg⁸-substitution. GnRH I (● and ○); chicken GnRH I ([Gln⁸]GnRH) (■ and □); frog GnRH ([Trp⁸]GnRH) (◆ and ◇); seabream GnRH ([Ser⁸]GnRH) (▲ and △). -----, wild-type; —, N7.45A. Arrows indicate shift in affinity of the mutant receptor for Arg⁸-substituted GnRHs at the N7.45A mutant receptor.

TABLE 1

The binding of GnRH analogues to wild-type and mutant human GnRH receptors

Ligand binding were conducted as described under "Experimental Procedures." Values are mean \pm S.E. from three or more independent experiments. The competing radioligand was 125 I-[His⁵,D-Tyr⁶]GnRH.

	Binding affinity (IC ₅₀)					
	GnRH I	GnRH II	[Tyr ⁸]GnRH	[Gln ⁸]GnRH	[Trp ⁸]GnRH	[Ser ⁸]GnRH
	<i>nm</i>	<i>nm</i>	<i>nm</i>	<i>nm</i>	<i>nm</i>	<i>nm</i>
Wild-type	2.6 \pm 0.2	29 \pm 2	222 \pm 13	80 \pm 6	111 \pm 8	684 \pm 43
Wild-type + IN3 ^a	2.5 \pm 0.3	28 \pm 3	211 \pm 23	94 \pm 10	107 \pm 5	621 \pm 30
N7.45A ^a	1.5 \pm 0.2	3.7 \pm 0.5	16 \pm 3	8.1 \pm 2.2	12 \pm 1	48 \pm 3
C6.47A ^a	3.2 \pm 0.1	9.6 \pm 0.7				
C6.47Y ^a	2.8 \pm 0.3	5.2 \pm 0.6				

^a With IN3 pretreatment.FIGURE 3. GnRHs elicited PhI responses at wild-type and N7.45A mutant receptors. A, GnRH I (● and ○) and GnRH II (■ and □) stimulated PhI responses. B, PhI responses elicited by GnRHs from other species with Arg⁸-substitution, ●, GnRH I; ■ and □, chicken GnRH I ([Gln⁸]GnRH); ◆ and ◇, frog GnRH ([Trp⁸]GnRH); ▲ and △, seabream GnRH ([Ser⁸]GnRH). -----, wild-type; ----, N7.45A.

Identification of the Asn^{7.45}-interacting Residue in the Human GnRH Receptor—In the refined GnRH receptor homology model in the inactive state, built on the crystal structure of bovine rhodopsin in the dark state (14), Asn^{7.45} faces toward Cys^{6.47}. Mutation of Cys^{6.47} to Ala or to Tyr (a naturally occurring mutant in human infertility) led to undetectable ligand binding and PhI responses. This phenotype is similar to that of N7.45A. The mutant receptor expression of C6.47A and C6.47Y at the cell surface was rescued by IN3 pretreatment, giving 38 and 18% that of the wild-type (Fig. 4B, inset). As with N7.45A, the mutations C6.47A and C6.47Y led to 3–5-fold increases in GnRH II binding affinity as compared with the wild-type receptor (Fig. 4A and Table 1). Both mutant receptors showed an increased signaling efficacy for GnRH II, yielding E_{max} responses greater than that of the wild-type with decreased EC₅₀ values (Fig. 4B and Table 2).

DISCUSSION

GPCRs for peptide ligands are frequently present as a variety of subtypes encoded by distinct genes. The presence of multiple isoforms of peptide ligands which preferentially bind to the cognate receptor subtypes implies co-evolution of peptide ligands and receptors. The conventional wisdom in regard to ligand binding and effector coupling selectivity is that receptor subtypes incorporate changes in binding site residues for ligand selectivity and changes in intracellular domains for coupling selectivity. Our studies here have shown an important role of receptor conformations in determining ligand binding selectivity and signaling efficacy in the human GnRH receptor.

Mutation of Asn^{7.45} to Ala led to undetectable ligand binding and PhI responses, which were rescued by a membrane-permeant, non-peptide GnRH antagonist, IN3 (Fig. 2, A and B), suggesting that the side chain of Asn^{7.45} makes intramolecular interactions which are important for receptor folding. Disruption of the intramolecular interactions appears to cause receptor conformational changes which can be modulated by the pharmacological chaperon, facilitating mutant receptor trafficking to the cell surface and indicating a ligand influence on receptor con-

formations. The pharmacological chaperoning effects of IN3 have been extensively studied by Conn and co-workers (27–30) in which IN3 has been shown to increase protein expression levels of mutant receptors on the cell membranes and to facilitate trafficking of the misfolded mutant receptors from endoplasmic reticulum (ER) to the cell surfaces. Membrane-permeant antagonists have also been extensively used to rescue receptor expression of structurally unstable mutants on cell surfaces in other GPCRs (31–39).

The mutation N7.45A had no or only a marginal effect on the receptor binding affinity for GnRH I, [His⁵]GnRH and [Trp⁷]GnRH, but markedly increased receptor binding affinity for GnRH II and [Tyr⁸]GnRH (Fig. 2, B and C). This is consistent with our previous suggestion that Tyr⁸ in GnRH II is involved in receptor conformational selection (10). An allosteric effect of Asn^{7.45} mutation on ligand binding affinity was also observed in the M₁ muscarinic acetylcholine receptor (mAChR), which increased receptor binding affinity not only for agonists, but also for certain antagonists (16).

The conformation of the wild-type human GnRH receptor has apparently evolved for high affinity binding to mammalian GnRH I, which contains Arg⁸ and therefore has a lower affinity for the second endogenous ligand, GnRH II, possessing Tyr⁸ (Fig. 2B) and GnRHs from other species, which only have one amino acid difference in position 8, including chicken GnRH I ([Gln⁸]GnRH), frog GnRH ([Trp⁸]GnRH), and seabream GnRH ([Ser⁸]GnRH) (Fig. 2D). Most interestingly, mutation of Asn^{7.45} to Ala also markedly increased the

TABLE 2

Receptor expression and functional responses of the wild-type and mutant GnRH receptors

Measurements of the receptor expression levels on cell surfaces (B_{\max}) using radioligand binding assay on whole cells, and PhI responses were conducted as described under "Experimental Procedures." The B_{\max} and the maximum PhI responses (E_{\max}) were expressed relative to a wild-type control in each transfection. Values are mean \pm S.E. from three or more independent experiments.

	B_{\max}	PhI responses									
		GnRH I		GnRH II		[Gln ⁸]GnRH		[Trp ⁸]GnRH		[Ser ⁸]GnRH	
		EC ₅₀	E_{\max}	EC ₅₀	E_{\max}	EC ₅₀	E_{\max}	EC ₅₀	E_{\max}	EC ₅₀	E_{\max}
	%	nM	%	nM	%	nM	%	nM	%	nM	%
Wild-type	100	0.2 \pm 0.1	100	6.9 \pm 1.4	100	10 \pm 2	83 \pm 9	4.6 \pm 0.6	53 \pm 6	48 \pm 6	44 \pm 5
N7.45A ^a	40 \pm 4	0.2 \pm 0.1	110 \pm 13	2.2 \pm 0.4	122 \pm 7	5.1 \pm 1.7	132 \pm 12	8.5 \pm 0.3	104 \pm 5	50 \pm 4	102 \pm 4
C6.47A ^a	38 \pm 3	0.1 \pm 0.1	122 \pm 8	1.8 \pm 0.9	131 \pm 9						
C6.47Y ^a	18 \pm 3	0.2 \pm 0.1	102 \pm 7	2.2 \pm 1.2	115 \pm 6						

^a With IN3 pretreatment.

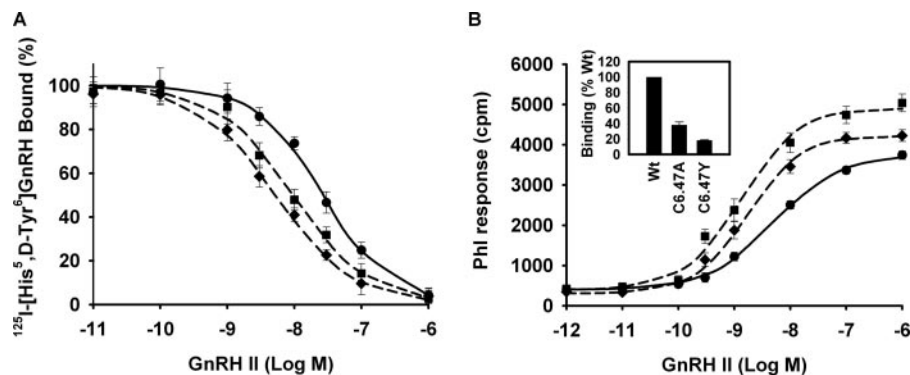


FIGURE 4. Binding and PhI assays of GnRH II at wild-type, C6.47A and C6.47Y mutant receptors. A, competitive binding showing an increased affinity of the mutant receptors for GnRH II. B, PhI assay of GnRH II. ●, wild-type; ■, C6.47A; ◆, C6.47Y. Inset shows the mutant receptor expressions relative to the wild-type level.

mutant receptor affinity for chicken GnRH I, frog GnRH, and seabream GnRH, as that of GnRH II and [Tyr⁸]GnRH. These results indicate an important role of receptor conformations in determining ligand binding selectivity developed during evolution, which can be manipulated by mutation-induced receptor conformational changes without alteration of the side chains of the ligand binding sites. These results support our proposal of co-evolution of ligand-receptor conformations. A single amino acid in position 8 of GnRHs acts as a structural determinant for receptor binding selectivity. We propose that ligands might exert a directive role in the evolution of receptor structure including primary and tertiary structures accounting for the origin of receptor specificity and diversity, consistent with the proposal that neuropeptide genes arose before the corresponding receptor genes and that their receptors might have evolved as targets for extant peptide ligands (40).

Chicken GnRH I ([Gln⁸]GnRH), frog GnRH I ([Trp⁸]GnRH), and seabream GnRH I ([Ser⁸]GnRH), whose side chains at position 8 appear to be able to make H-bonds with receptor contact residues, act as partial agonists in the wild-type human GnRH receptor (Fig. 3B). Most interestingly, all of them became full agonists in the N7.45A mutant receptor. We propose that GnRHs from different species that differ by only one amino acid in position 8 can selectively stabilize different receptor active conformations with different signaling efficacy. We envisage this occurs through common and differential receptor intramolecular and receptor-ligand intermolecular interactions. Arg⁸ of GnRH I (Fig. 1B) has been shown to

interact with Asp^{7.32} (Fig. 1C), but this is not the case for Tyr⁸ of GnRH II (41). There is increasing evidence that different ligands can induce different receptor conformations with different signaling capability (42, 43) and in such a way, some partial agonists and even some inverse agonists become full agonists in activating different signaling pathways (44–46). Interestingly, agonists differing by only a single hydroxyl group can lead to differential signaling in a *Drosophila* octopamine/tyramine receptor (47). Apparently,

high affinity ligand binding can be achieved not only by optimization of ligand binding sites, but also by inducing ligand-specific receptor intramolecular contacts that stabilize each binding partner (48), hence creating a ligand-specific receptor conformation which can be facilitated by weakening the receptor constraint networks. Recent studies have clearly shown that partial agonists stabilize a receptor conformation differing from that of full agonists in other GPCRs (49–51).

The marked loss of receptor binding (undetectable) without loss of binding affinity (determined after rescue with IN3) and the increases of signaling efficacy of the mutation of Asn^{7.45} to Ala indicate that the side chain of Asn^{7.45} makes intramolecular interactions, forming part of the receptor constraint network. To identify the residues interacting with Asn^{7.45}, we mutated residues (Glu^{2.53}, Ser^{3.35}, Cys^{6.47}, Trp^{6.48} (52), Thr^{6.49}, and Asp^{7.49}), which potentially interact with Asn^{7.45}, predicted by molecular modeling. Only Cys^{6.47} mutants gave a similar phenotype of unchanged binding affinity for GnRH I but increased binding affinity for GnRH II (Fig. 4) to the N7.45A mutant. Our molecular modeling has shown that the side chain of Asn^{7.45} can make an intramolecular interaction with Cys^{6.47} in the inactive state of the receptor (Fig. 5, A and B). The model was validated by accommodation of all experimentally identified receptor intramolecular interactions (10, 53–56) and the experimentally identified GnRH ligand-receptor intermolecular interactions (Fig. 1C) (6, 10). We therefore propose that the residues of Asn^{7.45} and Cys^{7.49} form part of the intramolecular constraint network involved in the stabilization of different

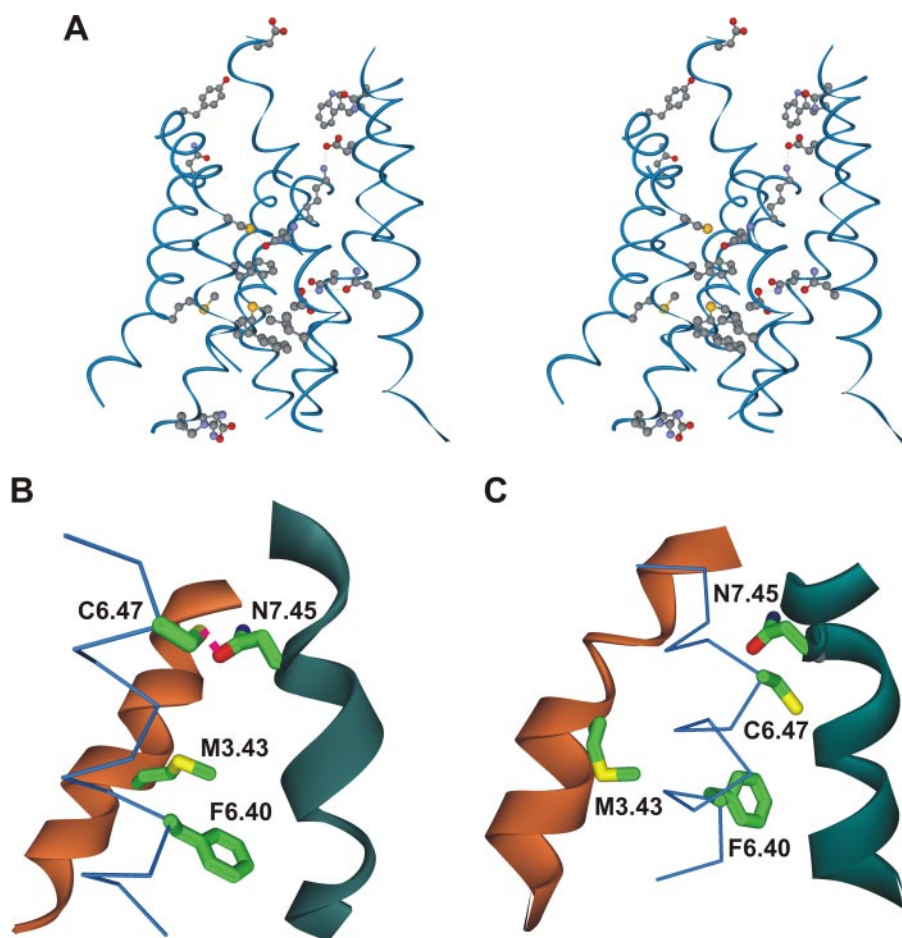


FIGURE 5. Homology models of human GnRH receptor in inactive and active conformations. *A*, stereo view of the 7-TM domains of the human GnRH receptor. The model was derived from the crystal structure of bovine rhodopsin in the inactive state (see "Experimental Procedures"). The model reveals the hydrogen bond interaction between Cys^{6.47} and Asn^{7.45}. The previously experimentally identified hydrogen bonds (green dash lines) between Asp^{2.61} and Lys^{3.32} (56), between Asp^{3.49} and Arg^{3.50} (55), and between Asn^{1.50}, Asn^{2.50}, and Asp^{7.49} (53, 54) and the hydrophobic interactions between Met^{3.43} and Phe^{6.40} (and surrounding residues Met^{5.54}, Phe^{6.44}, and Ile^{7.32}) (10) among the 7-TM domains, which validate our GnRH receptor model, were also shown. The GnRH receptor binding residues Asp^{2.61}, Trp^{2.64}, Asn^{2.65}, Lys^{3.32}, Asn^{5.39}, Tyr^{6.58}, and Asp^{7.32} (see Fig. 1C) are also included. *B*, intramolecular interactions between Cys^{6.47} and Asn^{7.45}. The side chains of Cys^{6.47} and Asn^{7.45} form part of allosteric intramolecular communication networks that confer GnRH ligand binding selectivity and signaling efficacy. Two previously identified residues (Met^{3.43} and Phe^{6.40}) whose mutations have no effect on GnRH I binding affinity, but specifically increase affinity for GnRH II and [Tyr⁸]GnRH are also shown (10). *C*, a GnRH receptor model in the active conformation, in which there are no intramolecular interactions between Cys^{6.47} and Asn^{7.45} and between Met^{3.43} and Phe^{6.40}. TM 3 is shown in orange, TM 6 in blue, and TM 7 in olive green in *B* and *C*.

receptor conformations which have preferential engagement with partial and full agonists. Partial agonists may only break part of the intramolecular constraint network. Mutations of Cys^{6.47} in the β_2 adrenergic receptor (57) and Asn^{7.45} in the histamine H₁ receptor (58) lead to constitutive activation of the receptors, indicating an important role of this residue in the receptor conformational switch. We have built a model of the human GnRH receptor in an active conformational state using the crystal structure of a photoactivated deprotonated intermediate of bovine rhodopsin (15) as a template. In the model, the intramolecular interactions between Cys^{6.47} and Asn^{7.45}, and between Met^{3.43} and Phe^{6.40}, which we identified previously (10), are disrupted due to a motion of the middle section of TM 3, as seen as a disorder of the helix (Fig. 5C), and a slight outward movement of TM 6 followed by a small clockwise rotation (viewed from the extracellular surface) of the intramolecular

segment by using Pro^{6.50} as a hinge (59) (Fig. 5C). Consistent with the mutagenesis results, our molecular modeling also indicates that these intramolecular interactions are involved in receptor conformational transition. Interestingly, no constitutive activity in any mutations of the human GnRH receptor was observed, unlike the β_2 (57) and H₁ (58) receptors. This indicates that weakening the intramolecular interactions in the human GnRH receptor is not sufficient to obtain an active conformation, but rather modifies receptor conformational states which are, at least, allosterically involved in ligand binding selectivity and signaling efficacy. We propose that GnRH ligand-induction of new receptor intra- and intermolecular interactions might be a fundamental component for GnRH receptor activation, rather than a ligand-induced disruption of the receptor intramolecular constraint networks, which we proposed as a mechanism of the M₁ mAChR activation (16). This may provide an explanation for the distinct pharmacological profiles of GnRH analogues in stimulating pituitary gonadotropin and inhibiting cancer cell proliferation (6). Of the endogenous ligands GnRH I is more potent in stimulating gonadotropins (6) but GnRH II has greater antiproliferative potency (60). Interestingly, the presence of Asp^{7.49}, located one helix below Asn^{7.45} in the

GnRH receptor because of a reciprocal exchange of the highly conserved Asp^{2.50}-Asn^{7.49} pair in other GPCRs prevents the GnRH receptor from coupling to phospholipase D via small G proteins (61), supporting our conclusion that residues within this region play an important role in the stabilization of different receptor conformations, and account for ligand binding and signaling selectivity.

In conclusion, our molecular modeling and mutagenesis studies have indicated that the side chains of the highly conserved Asn^{7.45} and Cys^{6.47} make intramolecular contacts in the inactive state (Fig. 5, *A* and *B*) which form part of the receptor allosteric network, coupling to specific structural elements of the GnRH analogues. This may underlie different receptor activation mechanisms, creating different receptor active conformations with potential ligand selective signaling described for these ligands. The identification of structural elements for

ligand and receptor conformational selection could have implications for the development of novel ligands that selectively activate one signaling pathway, bypassing others, and hence with improved pharmacological specificity and profiles. Our studies also support our proposal that ligand binding selectivity is determined not only by ligand binding residues, but also by receptor conformation. The conformation of GPCRs has been specialized during evolution by forming a complex receptor intramolecular interaction network. This accounts for selective binding of the cognate ligands and G proteins. The highly conserved amino acids appear to form part of the allosteric network which might serve as constraints for receptor inactive states. Mutation of a residue within the allosteric network can alter receptor binding selectivity of ligands and G proteins through subtle receptor conformational changes, which might be one of the mechanisms of development of ligand binding and signaling selectivity and diversity of GPCRs during evolution.

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A Crucial Role for $G\alpha_{q/11}$, But Not $G\alpha_{i/o}$ or $G\alpha_s$, in Gonadotropin-Releasing Hormone Receptor-Mediated Cell Growth Inhibition

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GnRH acts on its cognate receptor in pituitary gonadotropes to regulate the biosynthesis and secretion of gonadotropins. It may also have direct extrapituitary actions, including inhibition of cell growth in reproductive malignancies, in which GnRH activation of the MAPK cascades is thought to play a pivotal role. In extrapituitary tissues, GnRH receptor signaling has been postulated to involve coupling of the receptor to different G proteins. We examined the ability of the GnRH receptor to couple directly to $G\alpha_{q/11}$, $G\alpha_{i/o}$, and $G\alpha_s$, their roles in the activation of the MAPK cascades, and the subsequent cellular effects. We show that in $G\alpha_{q/11}$ -negative cells stably expressing the GnRH receptor, GnRH did not induce activation of ERK, jun-N-terminal kinase, or P38 MAPK. In contrast to $G\alpha_i$ or chimeric $G\alpha_{qi5}$, transfection of $G\alpha_q$ cDNA

enabled GnRH to induce phosphorylation of ERK, jun-N-terminal kinase, and P38. Furthermore, no GnRH-mediated cAMP response or inhibition of isoproterenol-induced cAMP accumulation was observed. In another cellular background, [35 S]GTP γ S binding assays confirmed that the GnRH receptor was unable to directly couple to $G\alpha_i$ but could directly interact with $G\alpha_{q/11}$. Interestingly, GnRH stimulated a marked reduction in cell growth only in cells expressing $G\alpha_q$, and this inhibition could be significantly rescued by blocking ERK activation. We therefore provide direct evidence, in multiple cellular backgrounds, that coupling of the GnRH receptor to $G\alpha_{q/11}$, but not to $G\alpha_{i/o}$ or $G\alpha_s$, and consequent activation of ERK plays a crucial role in GnRH-mediated cell death. (*Molecular Endocrinology* 22: 2520–2530, 2008)

MAMMALIAN HYPOTHALAMIC GnRH (termed GnRH-I) is a decapeptide hormone that plays key roles in the regulation of reproduction. It is synthesized in the hypothalamus and transported in the hypothalamo-hypophyseal portal circulation to the anterior pituitary. Here it binds to its cognate receptor, a member of the seven-transmembrane G protein-coupled receptor (GPCR) superfamily, and stimulates the biosynthesis and secretion of LH and FSH (1). As well as having a key role in reproductive behavior, evidence suggests that GnRH may act peripherally, via an autocrine/paracrine mechanism, to exert a growth-regulatory effect on certain cell types (2). Indeed, GnRH and the GnRH receptor have been found in extrapituitary tissues such as the ovary (3) and the mammary gland (4). Cancers of the breast (5–7), ovary (6, 8), endometrium (9), and prostate (10) have also been

shown to express both the ligand and the receptor. Additionally, several studies, both *in vitro* and *in vivo*, have demonstrated that direct application of GnRH analogs to receptor-expressing cancer cells results in an attenuation of cellular proliferation and activation of proapoptotic signaling mechanisms (11–19). In many of these cases, activation of the MAPK signaling cascades is thought to play a fundamental role (16, 17, 20, 21).

Several groups have demonstrated that GnRH stimulates phosphorylation of ERK, jun-N-terminal kinase (JNK), and P38, three prominent members of the MAPK superfamily, in α T3-1 and L β T2 gonadotrope cell lines and a wide variety of GnRH receptor transfected cells (21–25). How these cascades are initiated upstream by the activated receptor and which of them impinge on cell growth inhibition remains unclear. It has been proposed that whereas the actions of GnRH at the pituitary are mediated by interactions of the receptor with G proteins of the $G\alpha_{q/11}$ subfamily and consequent signaling to and activation of, among other molecules, ERK, JNK and P38 (21, 24, 26), the antiproliferative actions of GnRH are best explained via an interaction of the receptor with $G\alpha_{i/o}$ (17, 27–30). Resultant induction of apoptosis coincident with phosphorylation of JNK (16) or other members of the MAPK

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Abbreviations: GPCR, G protein-coupled receptor; JNK, jun-N-terminal kinase; PKC, protein kinase C; PLC β , phospholipase C β ; SPA, scintillation proximity assay.

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superfamily (17, 20) has also been observed. It is plausible to speculate that interaction of the receptor with different G proteins may explain the published diversity of the mechanisms involved in MAPK activation and the subsequent effects on cellular fate. This hypothesis, however, is not uncontested (26). Indeed, despite circumstantial evidence, direct proof of the activation of multiple G proteins by the agonist-bound GnRH receptor is still missing.

To better understand the pathways involved in GnRH-mediated cell growth inhibition, we set out to study the G protein coupling profile of the GnRH receptor. We provide direct evidence, in multiple cellular backgrounds, that the receptor couples to G proteins of the $G_{\alpha_{q/11}}$ subfamily but not to $G_{\alpha_{i/o}}$ or G_{α_s} as previously suggested. We also demonstrate that ERK, JNK, and P38 activation in response to GnRH treatment may be mediated by the activation of $G_{\alpha_{q/11}}$. Furthermore, we show that $G_{\alpha_{q/11}}$ may facilitate the induction of proapoptotic signaling by GnRH and that the ERK cascade, but neither the JNK nor the P38 pathway, plays a pivotal role.

RESULTS

GnRH-Stimulated MAPK Phosphorylation May Be Mediated by $G_{\alpha_{q/11}}$

To facilitate potential coupling of the GnRH receptor to G proteins other than $G_{\alpha_{q/11}}$, we stably transfected the receptor into $G_{\alpha_{q/11}}$ -negative MEF cells. These cells thus eliminate potential competition from $G_{\alpha_{q/11}}$ for binding of the receptor. The MEF S19 cell line expresses more receptors per cell than either the SCL60 or the $L\beta T2$ cells, although the binding affinity of GnRH-I is not significantly different (MEF S19, 11.1 ± 2.2 nM; SCL60, 10.1 ± 1.7 nM; and $L\beta T2$, 10.1 ± 1.3 nM) (Fig. 1). The higher expression of the GnRH receptor in these cells is reflected by their larger size. In addition, the estimated number of GnRH receptor binding sites on $\alpha T3-1$ cells is approximately 50% of the number on primary gonadotropes (31). Thus, the MEF S19 cells may better reflect GnRH receptor expression levels encountered *in vivo*. To elucidate the ability of GnRH to activate ERK, JNK, and P38 in these cells, GnRH-I was applied in both a time- and dose-dependent manner (Fig. 2, A and B, respectively). GnRH-I stimulation brought about no significant increase in the levels of pERK1/2, pJNK1, or pP38 at any time or dose tested when compared with vehicle-treated controls. Transient expression of G_{α_q} allowed GnRH-I to elicit an increase in ERK1/2 phosphorylation with a maximal response of approximately 3-fold that of controls after 5 min stimulation. Additionally, JNK1 and P38 phosphorylation became evident, giving maximal responses of approximately 3.5-fold that of controls after 5 min stimulations and 30 min stimulations, respectively. Agonist dose-response analysis yielded EC_{50} values for the induction of pERK1/2,

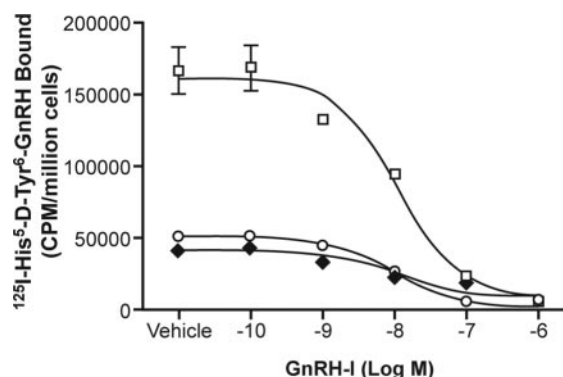


Fig. 1. GnRH Receptor Expression in the MEF S19, SCL60, and $L\beta T2$ Cell Lines as Measured by Radioligand Binding

Intact MEF S19 (\square), SCL60 (\circ), and $L\beta T2$ (\blacklozenge) cells were incubated with ^{125}I -labeled [$His^5, D-Tyr^6$] GnRH (100,000 cpm/0.5 ml/well) and either vehicle (0.2% propylene glycol) or various concentrations of unlabeled GnRH-I as indicated for 4 h at 4 C. After incubation, cells were rapidly washed twice with cold PBS and solubilized in 0.1 M NaOH. Radioactivity was counted by γ -spectrometry. Data are representative of at least three independent experiments and the mean counts per minute per million cells (CPM/million cells) \pm SE is presented.

pJNK1, and pP38 after 10 min stimulation of 0.95, 3.32, and 2.36 nM GnRH-I, respectively. In untransfected cells, the complete absence of $G_{\alpha_{q/11}}$ was verified both by Western blotting and lack of phosphoinositide hydrolysis (Fig. 2, C and D, respectively). Additionally, when compared with $L\beta T2$ cells, the levels of $G_{\alpha_{q/11}}$ after transient G protein transfection were within the physiological range.

The GnRH Receptor Does Not Directly Interact with $G_{\alpha_{i/o}}$ or G_{α_s}

Thus far we have confirmed that transfection of G_{α_q} allows the detection of significant activation of ERK, JNK, and P38 in response to GnRH treatment. Nevertheless, several studies have argued that these responses may also be mediated by interactions of the GnRH receptor with other G proteins. Although the above results indicate that in the complete absence of $G_{\alpha_{q/11}}$, it was not possible to detect significant increases in MAPK activation when compared with control cells, we attempted to address this theory. Initially, we considered the possibility that transient overexpression of G_{α_i} could facilitate detectable MAPK signaling. In contrast to G_{α_q} , transient transfection of each of the three subtypes of G_{α_i} did not enable GnRH-I to significantly increase ERK1/2, JNK1, or P38 phosphorylation when compared with controls (Fig. 3A). Additionally, we made use of a chimeric G protein, $G_{\alpha_{q15}}$. The C terminus of the G protein α -subunit has been shown to play a pivotal role in defining the specificity of receptor-G protein interactions. Mutation of the last five C-terminal amino acids is sufficient to completely switch receptor coupling selectivity (32).

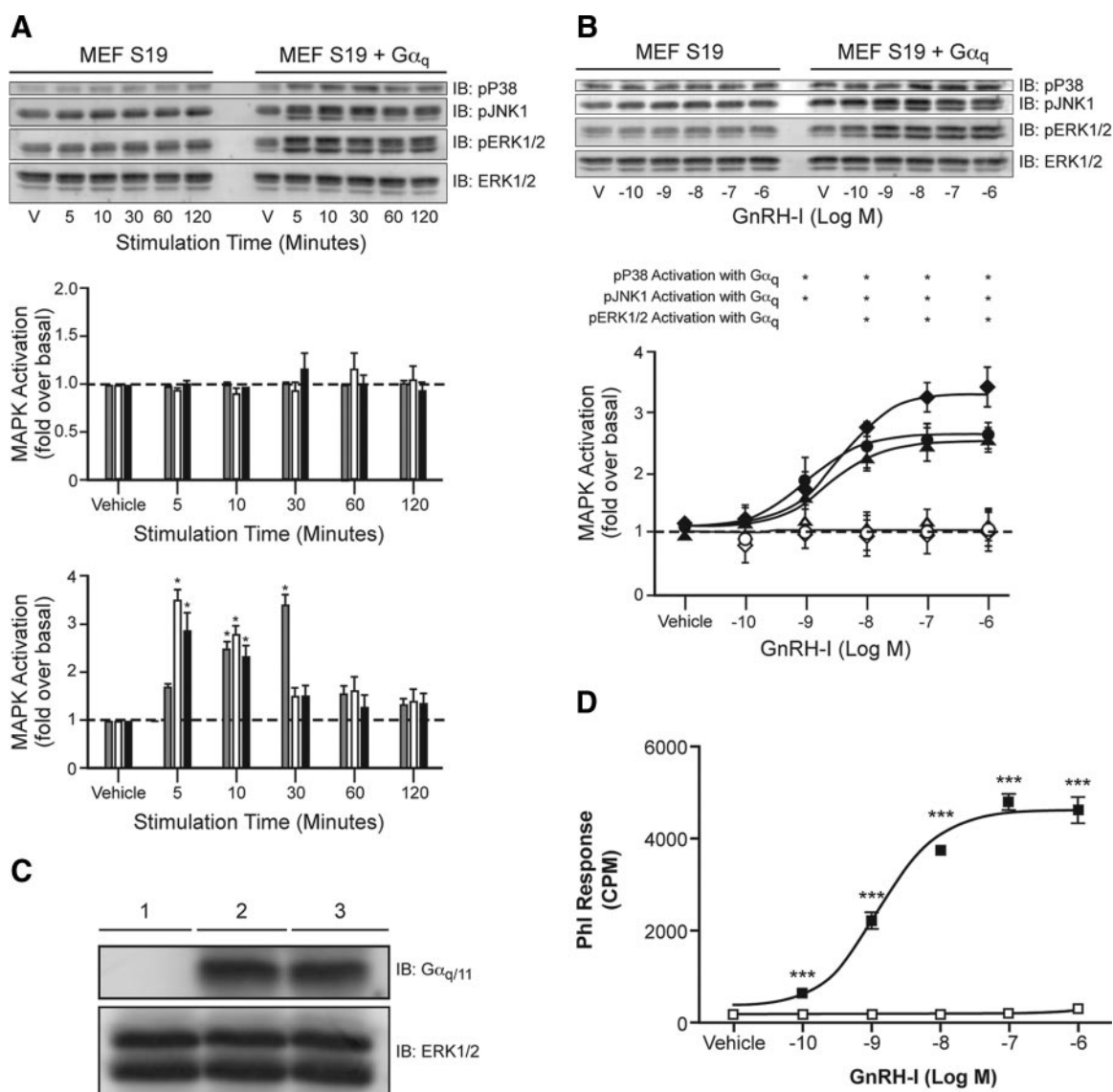


Fig. 2. Immunoblots Depicting the Time and Dose Dependence of GnRH-I-Induced, $G\alpha_q$ -Mediated Phosphorylation of ERK1/2, JNK1, and P38

A, Time course of GnRH-I-stimulated MAPK phosphorylation. MEF S19 cells transiently transfected with vector or $G\alpha_q$ cDNA were serum starved for 16 h before being treated with vehicle (0.2% propylene glycol; V) or 1 μ M GnRH-I for the indicated times. Representative blots are shown. Data from at least three independent experiments were quantified (using total ERK1/2 as a loading control), and the mean fold over control \pm SE for the activation of ERK1/2 (black bars), JNK1 (white bars), and P38 (gray bars) in cells transfected with vector (middle panel) or $G\alpha_q$ (bottom panel) cDNA is presented below the corresponding blots. *, $P < 0.05$, representing statistical significance from vehicle-treated controls. **B**, Dose response of GnRH-I-stimulated MAPK phosphorylation. MEF S19 cells transiently transfected with vector or $G\alpha_q$ cDNA were serum starved for 16 h before being treated with vehicle (0.2% propylene glycol; V) or increasing doses of GnRH-I (0.1, 1, 10, and 100 nM and 1 μ M) as indicated for 10 min. Representative blots are shown. Data from at least three independent experiments were quantified (using total ERK1/2 as a loading control), and the mean fold over control \pm SE for the activation of ERK1/2 (\circ and \bullet), JNK1 (\diamond and \blacklozenge), and P38 (\triangle and \blacktriangle) in cells transfected with vector (open symbols) or $G\alpha_q$ (filled symbols) cDNA is presented below the corresponding blots. *, $P < 0.05$, representing statistical significance from vehicle-treated controls. **C**, Immunoblots depicting the relative expression of $G\alpha_q$. MEF S19 cells transiently transfected with vector (lane 1) or $G\alpha_q$ (lane 2) cDNA and L β T2 cells (lane 3) were serum starved for 16 h. Unstimulated cell lysates were collected and used to verify transfection efficiency and relative G protein expression level. Representative blots are shown. **D**, Phosphoinositide hydrolysis assays depicting the functionality of transfected $G\alpha_q$. MEF S19 cells transiently transfected with vector (\square) or $G\alpha_q$ (\blacksquare) cDNA were labeled overnight with 1 μ Ci/ml myo-D-[3 H]inositol before being treated with vehicle (0.2% propylene glycol; V) or increasing doses of GnRH-I (0.1, 1, 10, and 100 nM and 1 μ M) as indicated for 30 min. The [3 H]inositol phosphates were processed as described in *Materials and Methods* and quantified by liquid scintillation counting. Data are representative of at least three independent experiments, and the mean counts per minute (CPM) \pm SE is presented. ***, $P < 0.001$, representing statistical significance from vehicle-treated controls.

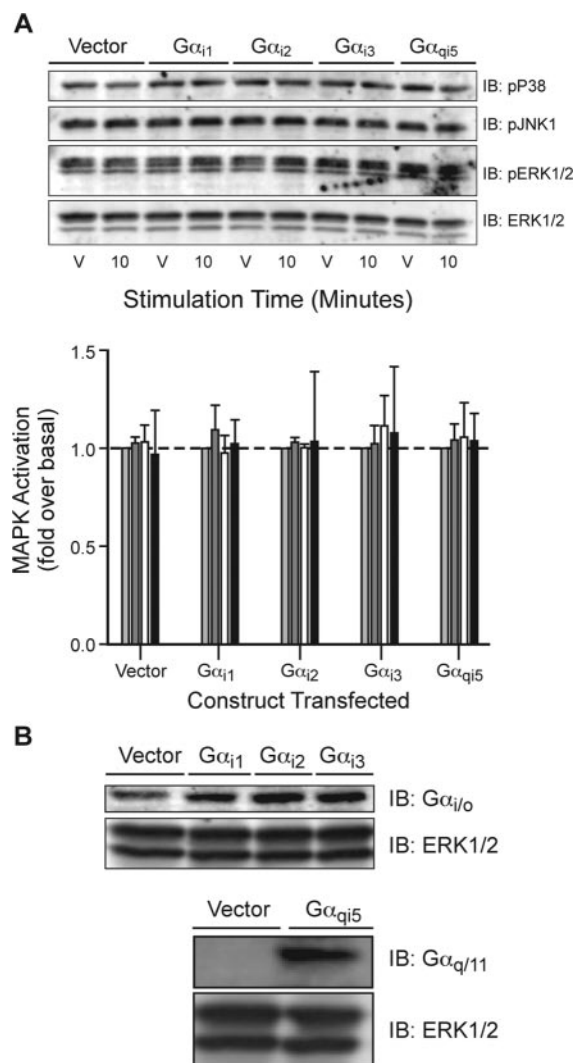


Fig. 3. Immunoblots Depicting the Lack of Effect of Transient Overexpression of Gα_{i1-3} or Gα_{qi5} on GnRH-I-Induced Phosphorylation of ERK1/2, JNK1, and P38

A, MEF S19 cells transiently transfected with vector, Gα_{i1}, Gα_{i2}, Gα_{i3}, or Gα_{qi5} cDNA were serum starved for 16 h before being treated with vehicle (0.2% propylene glycol; V) or 1 μM GnRH-I for 10 min. Representative blots are shown. Data from at least three independent experiments were quantified (using total ERK1/2 as a loading control), and the mean fold over control ± SE for the activation of ERK1/2 (black bars), JNK1 (white bars), and P38 (dark gray bars) relative to vehicle-treated controls (light gray bars) is presented below the corresponding blots. B, MEF S19 cells transiently transfected with vector, Gα_{i1}, Gα_{i2}, Gα_{i3}, or Gα_{qi5} cDNA were serum starved for 16 h. Unstimulated cell lysates were collected and used to verify transfection efficiency and relative G protein expression level. Representative blots are shown.

The cDNA of Gα_{qi5} codes for a G protein α-subunit consisting of mainly Gα_q. The last five C-terminal amino acids have, however, been substituted for the corresponding amino acids from Gα_{i2}. This facilitates Gα_{i/o}-coupled GPCR signaling through phospholipase C_β (PLC_β), and as such, a classical Gα_q-mediated

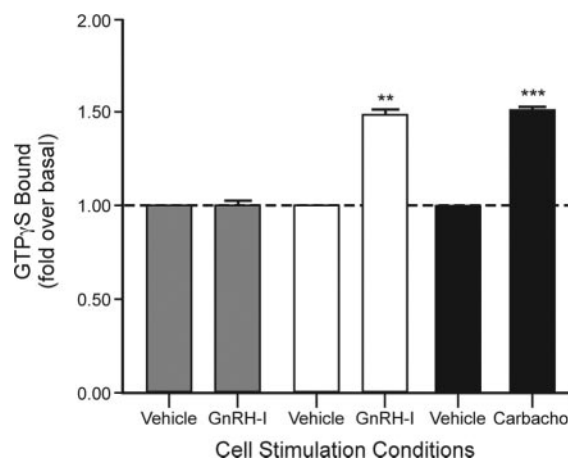


Fig. 4. Scintillation Proximity Assays Quantifying Receptor-Mediated Activation of Gα_{q/11} and Gα_i

Freshly prepared SCL60 (gray and white bars) and CHO-M₂ (black bars) membranes were incubated in a GTPγS assay buffer in the presence of vehicle (0.2% propylene glycol), 1 μM GnRH-I, or 10 μM carbachol and 200 pM [³⁵S]GTPγS for 1 h at 25 °C. Thereafter, antibodies against Gα_{i1/2} and Gα_{i3} (gray and black bars) or Gα_{q/11} (white bars) and anti-IgG-coated SPA beads were added and processed as described in Materials and Methods. Data from at least three independent experiments were quantified, and the mean fold over control ± SE for GTPγS bound is presented. **, P < 0.01; and ***, P < 0.001, representing statistical significance from vehicle-treated controls.

output becomes indicative of receptor coupling to Gα_{i/o}. Transient expression of Gα_{qi5} in the MEF S19 cell line did not enable the activated receptor to elicit a significant increase in ERK1/2, JNK1, or P38 phosphorylation when compared with control cells. Transfected cells showed significantly increased expression of Gα_{i1-3} and Gα_{qi5} (Fig. 3B).

To address the possibility that the GnRH receptor does interact with Gα_{i/o} but that Gα_{i/o} does not activate the MAPK cascades thus making it impossible to identify such interactions using MAPK phosphorylation as an output, we assessed the ability of GnRH to increase GTPγS binding to Gα_i and to inhibit intracellular cAMP accumulation. Furthermore, for the GTPγS experiments, we used a different cell line to investigate cell-type-specific differences. Stimulation of SCL60 cells with GnRH-I had no effect on GTPγS binding to Gα_i (Fig. 4). In contrast, stimulation of CHO-M₂ cells with carbachol significantly increased GTPγS-Gα_i binding, thereby providing a positive control for the Gα_i-GTPγS assay system. Stimulation of SCL60 cells with GnRH-I significantly increased GTPγS binding to Gα_{q/11} to approximately 1.5-fold that of controls. Moreover, stimulation of MEF S19 cells with isoproterenol increased intracellular cAMP levels to approximately 15-fold that of vehicle-treated controls. Costimulation of the same cells with isoproterenol and GnRH-I did not significantly affect the cAMP response obtained (Fig. 5). Significantly, stimulation with GnRH-I alone did not induce any increase in intracellular cAMP. Similar

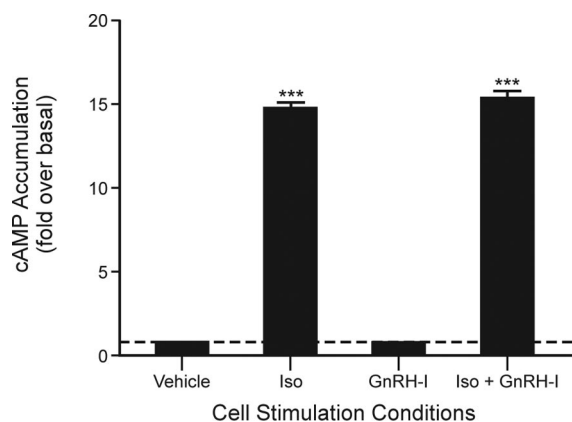


Fig. 5. cAMP Assays Determining the Effects of GnRH-I and Isoproterenol on Intracellular cAMP Accumulation

MEF S19 cells were treated with vehicle (0.2% propylene glycol), 3 μ M isoproterenol (Iso), 1 μ M GnRH-I, or 1 μ M GnRH-I and 3 μ M isoproterenol together for 30 min as indicated after a 30-min incubation with 1 mM 3-isobutyl-1-methylxanthine. Intracellular cAMP levels were measured as described in *Materials and Methods*. Data from at least three independent experiments were quantified, and the mean fold over control \pm SE is presented. ***, $P < 0.001$, representing statistical significance from vehicle-treated controls.

results were observed in COS-7 cells transiently expressing the GnRH receptor (White, C. D., and Z.-L. Lu, unpublished observations).

Coupling of the GnRH Receptor to $G_{\alpha_{q/11}}$ and Consequent Activation of the ERK Pathway Play a Crucial Role in GnRH-Mediated Cell Growth Inhibition

Continuous treatment of MEF S19 cells with GnRH-I resulted in a time- and dose-dependent inhibition of cell growth relative to vehicle-treated controls only in cells transfected with G_{α_q} (Fig. 6A). In these cells, significant growth inhibition was evident on d 1 (cell growth was $93.7 \pm 1.2\%$ that of controls) and increased in a time-dependent manner to reach a maximal inhibition on d 4 (cell growth was $69.8 \pm 2.8\%$ that of controls). SB203580 (a P38 inhibitor) and SP600125 (a JNK inhibitor) were unable to significantly rescue this inhibition, but PD98059 (a MAPK kinase-1/2 inhibitor) significantly decreased the GnRH-I-induced effect (cell growth was $90.4 \pm 1.5\%$ that of controls on d 4). Agonist dose-response analysis yielded an IC_{50} value for the induction of cell growth inhibition on d 4 of 4.54 nM GnRH-I (Fig. 6B). Vector-transfected control cells showed no significant inhibition of growth in response to GnRH-I treatment, nor did any of the MAPK inhibitors have a significant effect. Similar results were obtained in the SCL60 cell line (Fig. 6C). Continuous treatment with GnRH-I resulted in a time- and dose-dependent inhibition of cell growth relative to vehicle-treated controls. Significant growth inhibition was evident on d 1 (cell growth was $87.6 \pm 0.4\%$ that of controls) and increased in a time-

dependent manner to reach a maximal inhibition on d 4 (cell growth was $57.3 \pm 1.2\%$ that of controls). SB203580 and SP600125 were unable to significantly rescue this inhibition, but PD98059 significantly decreased the GnRH-I-induced effect (cell growth was $81.0 \pm 1.5\%$ that of controls on d 4). Agonist dose-response analysis yielded an IC_{50} value for the induction of cell growth inhibition on d 4 of 6.21 nM GnRH-I (Fig. 6D). YM-254890 (a $G_{\alpha_{q/11}}$ inhibitor) completely eliminated the GnRH-I-induced inhibition of cell growth. Trypan blue exclusion confirmed that detached cells were dead (as measured by dye uptake when compared with either live cell suspensions or vector-transfected samples), and these dead cells were more numerous than in samples from vector-transfected controls (White, C. D., and Z.-L. Lu, unpublished observations).

DISCUSSION

Binding of GnRH to its receptor at the plasma membrane initiates a variety of intracellular signaling events with distinct physiological outcomes. Since the original observations of GnRH-induced cell growth inhibition on receptor-expressing cell lines (33), substantial effort has been directed toward delineating the precise mechanisms involved. Considerable evidence suggests that the signaling cascades involved in GnRH-mediated cell growth inhibition are distinct from those involved in regulating gonadotropin secretion (17, 30, 34), and one possibility to explain this divergent signaling is to hypothesize the inherent ability of the receptor to directly interact with multiple classes of G proteins. Multiplicity of G protein coupling has after all been successfully confirmed for several other GPCRs (35). However, as yet, no group has provided direct evidence of such interactions, and the frequently repeated paradigm of the ability of the GnRH receptor to directly interact with $G_{\alpha_{i/o}}$ and G_{α_s} has led to confusion regarding the mechanisms by which GnRH can inhibit cell growth. To critically address this issue, we studied the G protein coupling profile of the GnRH receptor in stably transfected HEK293 cells and MEF cells derived from $G_{\alpha_{q/11}}$ knockout mice (36, 37). Additionally, we investigated the roles of the various G proteins in the activation of ERK, JNK, and P38 and the downstream cellular effects of activation of these MAPK modules.

GnRH-mediated activation of ERK, JNK, and P38 has been extensively studied over the past two decades (21, 24). We and others have shown that phosphorylation of these proteins readily occurs upon GnRH stimulation of receptor-expressing cell lines and that this activation is dependent on PLC_{β} and protein kinase C (PKC) (22, 23, 38). These data thus suggest the involvement of $G_{\alpha_{q/11}}$ -mediated signaling events. The role of $G_{\alpha_{i/o}}$ in MAPK activation in response to GnRH has also been the subject of much investigation. ERK activation in α T3-1 cells has been suggested to depend on a dual mechanism involving $G_{\alpha_{q/11}}$ and $G_{\alpha_{i/o}}$ (39). Similarly, in Caov-3 ovarian cancer cells,

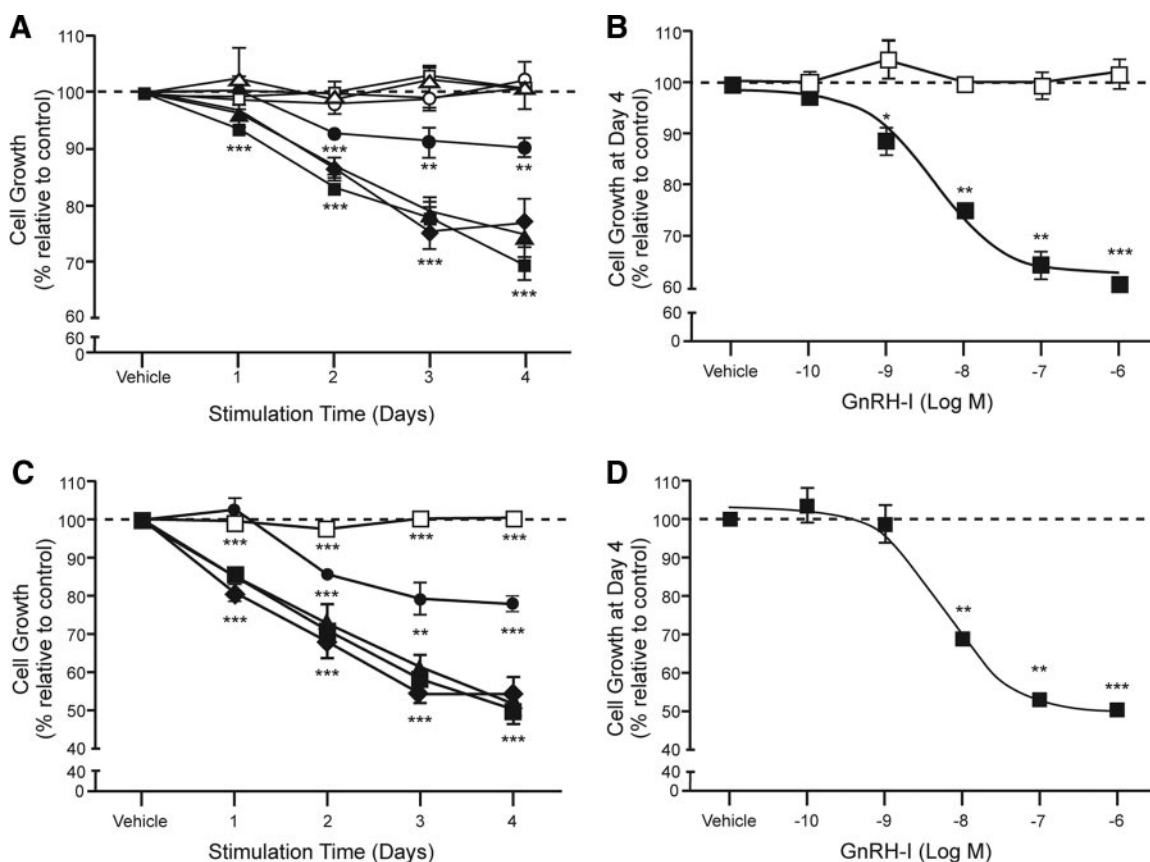


Fig. 6. Sulforhodamine B Assays Assessing the Effects of $G\alpha_q$ and the ERK, JNK, and P38 Pathways on GnRH-I-Induced Cell Growth Inhibition

A, Effect of $G\alpha_q$ and the MAPK pathways on GnRH-I-induced cell growth inhibition in MEF S19 cells. MEF S19 cells transiently transfected with vector (open symbols) or $G\alpha_q$ cDNA (filled symbols) were incubated in medium containing 10% serum with or without vehicle (dimethylsulfoxide; □ and ■), 50 μ M PD98059 (○ and ●), 50 μ M SP600125 (◇ and ◆), or 20 μ M SB203580 (△ and ▲) before being treated with vehicle (0.2% propylene glycol) or 100 nM GnRH-I for the indicated times. Data from at least three independent experiments were quantified, and the mean percent cell growth relative to control \pm SE is presented. **, $P < 0.01$; ***, $P < 0.001$, representing statistical significance from vector-transfected vehicle-treated controls (for cells transfected with $G\alpha_q$ and incubated in media containing dimethylsulfoxide) and from $G\alpha_q$ -transfected vehicle-inhibited controls (for cells incubated in medium containing 50 μ M PD98059). B, Dose dependence of GnRH-I-induced cell growth inhibition in MEF S19 cells. MEF S19 cells transiently transfected with vector (□) or $G\alpha_q$ cDNA (■) were incubated in medium containing 10% serum before being treated with vehicle (0.2% propylene glycol) or increasing doses of GnRH-I (0.1, 1, 10, and 100 nM and 1 μ M) as indicated for 4 d. Data from at least three independent experiments were quantified, and the mean percent cell growth relative to control at d 4 \pm SE is presented. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, representing statistical significance from vehicle-treated controls. C, Effect of $G\alpha_q$ and the MAPK pathways on GnRH-I-induced cell growth inhibition in SCL60 cells. SCL60 cells were incubated in medium containing 10% serum with or without vehicle (dimethylsulfoxide; □ and ■), 50 μ M PD98059 (○ and ●), 50 μ M SP600125 (◇ and ◆), 20 μ M SB203580 (△ and ▲), or 100 nM YM-254890 (□ and ■) before being treated with vehicle (0.2% propylene glycol) or 100 nM GnRH-I for the indicated times. Data from at least three independent experiments were quantified, and the mean percent cell growth relative to control \pm SE is presented. **, $P < 0.01$; ***, $P < 0.001$, representing statistical significance from vehicle-treated controls (for cells incubated in medium containing dimethylsulfoxide) and from vehicle-inhibited controls (for cells incubated in medium containing 50 μ M PD98059 or 100 nM YM-254890). D, Dose dependence of GnRH-I-induced cell growth inhibition in SCL60 cells. SCL60 cells were incubated in medium containing 10% serum before being treated with vehicle (0.2% propylene glycol) or increasing doses of GnRH-I (0.1, 1, 10, and 100 nM and 1 μ M) as indicated for 4 d. Data from at least three independent experiments were quantified, and the mean percent cell growth relative to control at d 4 \pm SE is presented. **, $P < 0.01$; ***, $P < 0.001$, representing statistical significance from vehicle-treated controls.

which have been shown to express GnRH receptor mRNA (although no evidence is given regarding expression at the protein level), ERK activation has been hypothesized as being mediated by a combination of interactions involving $G\alpha_{i/o}$ and $G\beta\gamma$ (20). Studies using pertussis toxin have also indicated a role for $G\alpha_{i/o}$

in the GnRH-induced phosphorylation of JNK and P38 (17). Interestingly, our data contradict these studies because we have found no evidence to support the theory of GnRH-induced activation of these MAPKs by $G\alpha_{i/o}$, even when $G\alpha_i$ is artificially overexpressed in our cell systems. We have, however, confirmed that $G\alpha_q$

$\alpha_{q/11}$ -mediated signaling plays a pivotal role in the GnRH receptor activation of the MAPKs.

Elegant studies have shown that coexpression of $G_{\alpha_{q15}}$ with several other GPCRs successfully enables $G_{\alpha_{i/o}}$ -coupled receptors to signal through PLC_{β} and PKC (40). It follows then that the inability of GnRH to induce phosphorylation of ERK, JNK, or P38 when $G_{\alpha_{q15}}$ is expressed suggests that the GnRH receptor does not directly interact with G proteins of the $G_{\alpha_{i/o}}$ subfamily. This conclusion is strengthened by the observation that GnRH does not inhibit isoproterenol-induced cAMP accumulation. However, by definition, the only direct measure of G protein activation by an activated receptor is receptor-catalyzed GDP/GTP exchange. Other analytical approaches yield only indirect results and the notion of GnRH receptor- $G_{\alpha_{i/o}}$ interaction has largely been derived from circumstantial evidence using pertussis toxin. In this case, our data, and the observations of Grosse and colleagues (26), which reported exclusive GnRH receptor-mediated labeling of $G_{\alpha_{q/11}}$ with [α - ^{32}P]GTP azidoanilide, show definitively that the GnRH receptor does not directly couple to $G_{\alpha_{i/o}}$ but does interact with $G_{\alpha_{q/11}}$. Reports by Shah and colleagues (41) using activated G proteins as an output support this interpretation. Here, stimulation of α T3-1 cells with GnRH agonists resulted in elevated agonist-induced down-regulation of $G_{\alpha_{q/11}}$ reflecting increased G protein turnover. $G_{\alpha_{i/o}}$ remained unaffected. Interestingly, it has recently been shown that G protein α -subunits can be activated directly by growth factor receptor tyrosine kinases (42). Thus, it is possible that GnRH receptor-mediated receptor tyrosine kinase transactivation could be responsible for previous interpretations implicating a direct interaction of the receptor with $G_{\alpha_{i/o}}$. Furthermore, the demonstration that very high agonist concentrations are required to facilitate GnRH receptor-mediated $G_{\alpha_{i/o}}$ signaling events (43) suggests that these events may potentially occur distally of the receptor-G protein interface.

In the early 1990s, Janovick and Conn (44) showed that treatment of rat pituitary cultures with cholera toxin increased LH release in response to GnRH treatment and interpreted this finding as being indicative that the GnRH receptor was directly coupled to G_{α_s} . Since then, many other groups have studied this possibility, but the results are inconclusive. The cAMP-protein kinase A pathway is important for gonadotrope function (45, 46); however, the involvement of G_{α_s} in this signaling cascade is still debated. Using palmitoylation as a measure of G protein activation, it was suggested that there was a direct interaction between the GnRH receptor and G_{α_s} in rat pituitary cells (47). This hypothesis was recapitulated in the $L\beta$ T2 cell line using cell-permeable peptides that uncouple G_{α_s} from the receptor (48). However, based on these and other observations, the Gudermann group (26) examined the possibility of a direct interaction between the activated receptor and G_{α_s} and, in agreement with our findings, failed to detect any G_{α_s} -mediated signal transduction in response to GnRH treatment. More recently, Larivière and colleagues identified a novel signaling pathway involving PKC δ and ϵ , which mediate

GnRH activation of a cAMP-sensitive promoter (49). These results, viewed collectively with our own data, question the possibility of a direct interaction between the GnRH receptor and G_{α_s} . Interestingly, it has also been shown that a dominant-negative mutant of G_{α_s} blocked not only G_{α_s} -mediated signaling from the calcitonin receptor but also that mediated by G_{α_q} (50) and that specific adenylate cyclase isoforms may be activated directly by $G\beta\gamma$ (51) or independently of G proteins altogether (52). It would therefore seem possible to conclude that signal transduction involving GnRH induction of the cAMP pathway remains to be fully elucidated but is unlikely to involve a direct interaction between the activated receptor and G_{α_s} . Indeed, given that we cannot detect any cAMP accumulation in response to GnRH in $G_{\alpha_{q/11}}$ -negative cells, taken together with the fact that the β_2 -adrenergic receptor agonist isoproterenol can elicit a marked increase in intracellular cAMP, we would suggest that the GnRH receptor-evoked small cAMP responses previously observed in certain cell lines are not mediated by the direct coupling of the receptor to G_s .

The MAPK pathways are evolutionarily conserved kinase cascades that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration, and apoptosis. Historically, ERK signaling is synonymous with cell proliferation (53), although the JNK and P38 pathways are regarded as being stress activated (54). Involvement of the JNK and P38 signaling cascades in GPCR-induced inhibition of cell proliferation has been widely documented (53–56), and here the GnRH receptor is no exception (16, 17, 21). In contrast to these data, we showed, in agreement with previous studies (20, 57), that P38 and JNK do not influence GnRH-induced cell growth inhibition. Additionally, we find that the ERK signaling pathway plays a critical role; inhibition of ERK activation significantly decreased cell growth inhibition in both cell lines tested.

ERK has been implicated in cell growth inhibition, cell cycle arrest, and the induction of proapoptotic signaling in a number of cell types (58, 59), and a large body of evidence indicates that the strength and duration of the ERK signal is kernel to determining cellular fate (19, 60, 61). Several studies have shown that strong and prolonged activation of ERK by constitutively active Ras or Raf leads to arrest in the G_1 phase of the cell cycle by inducing the expression of cyclin-dependent kinase inhibitors such as p53 and p21 (62, 63). As such, it seems plausible to speculate that continuous exposure of our cells to GnRH would result in ERK activation of a similar strength and duration. Thus, by inhibiting this pathway, this activation is abolished and GnRH-induced cell growth inhibition is significantly diminished. Interestingly, Zhang and colleagues (64) demonstrated that in $L\beta$ T2 cells, p53 is phosphorylated by GnRH in a P38-dependent manner. Although our results do not indicate a role for P38 in GnRH-mediated cell growth inhibition in the cell lines we have studied, it is possible that transformation of the $L\beta$ T2 cell line with SV40 large T antigen may influence the signaling pathways involved. Additionally,

MAPK activation in response to GnRH has also been suggested to be dependent on the cell background in which any studies are carried out (17, 21). Notably, however, some studies that report significant effects of JNK and P38 on GnRH-induced cell growth inhibition also imply the ability of the GnRH receptor to couple to $G_{\alpha_{i/o}}$ (17, 65). Perhaps, given our current findings, it would be prudent to directly verify these data.

The ability of many hormones and neurotransmitters to evoke diverse physiological and pathological responses, by binding to a single cognate receptor, brought about the hypothesis that one GPCR may have the inherent ability to activate multiple G proteins. As such, the differential effects of GnRH analogs at central and peripheral sites were thought to be mediated via interactions of the activated receptor with $G_{\alpha_{q/11}}$, $G_{\alpha_{i/o}}$, and G_{α_s} . In the present study, we provide direct evidence, in multiple cellular contexts, that the GnRH receptor does not couple to G proteins of the $G_{\alpha_{i/o}}$ or G_{α_s} subfamilies, that MAPK activation in response to GnRH treatment is entirely dependent, of the three G protein subtypes tested, on $G_{\alpha_{q/11}}$, and that the ERK pathway is significantly involved in GnRH-mediated cell death.

MATERIALS AND METHODS

Materials

The pMEP4 expression vector was kindly provided by Dr. Keith Leppard, University of Warwick, UK. PD98059 (50 μ M), SP600125 (50 μ M), and SB203580 (20 μ M) were all obtained from Calbiochem (Nottingham, UK). YM-254890 was kindly provided by Dr. Masatoshi Taniguchi, Astellas Pharma Inc., Japan. The $G_{\alpha_{q/11}}$ and $G_{\alpha_{i-3}}$ protein cDNAs were obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). The $G_{\alpha_{q15}}$ protein cDNA, the $G_{\alpha_{q/11}}$ -negative MEF cell line, and the $G_{\alpha_{i1/2}}$ and $G_{\alpha_{i3}}$ antibodies were kindly provided by Professor Graeme Milligan, University of Glasgow, Glasgow, UK. The $G_{\alpha_{q/11}}$ -negative MEF cell line was originally derived from a combined $G_{\alpha_{q/11}}$ double-knockout mouse and has been previously shown to have absolutely no endogenous $G_{\alpha_{q/11}}$ (36, 37). The CHO cell line stably expressing the M_2 -muscarinic receptor (designated CHO- M_2) was kindly provided by Professor Noel Buckley, University of Leeds (Leeds, UK). The L β T2 cell line was kindly provided by Dr. Pamela Mellon, University of California. [35 S]GTP γ S (1000–1250 Ci/mmol) and anti-IgG-coated scintillation proximity assay (SPA) beads were purchased from GE Healthcare (Buckinghamshire, UK), and the $G_{\alpha_{q/11}}$ (C-19) and $G_{\alpha_{i/o}}$ antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All other reagents were obtained from Sigma (Dorset, UK) unless otherwise stated.

Cell Culture and Transfection

Full-length GnRH receptor cDNA was subcloned into pMEP4 at *Not*I and *Xho*I and the construct transfected into $G_{\alpha_{q/11}}$ -negative MEF cells by electroporation. Cloned cells were selected using hygromycin resistance and screened by radioligand binding (Fig. 1) as previously described (66, 67). After appropriate selection, cells stably expressing the GnRH receptor (designated MEF S19) were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, appropriate antibiotics, and 50 μ g/ml hygromycin (Invitrogen Life Technologies, Paisley, UK) at 37 C in a humidified 5%

CO $_2$ atmosphere. Cells were seeded at a density of 1.0×10^5 cells/ml and allowed to grow in large culture flasks for 96 h before transfection. Transient transfections were performed by electroporation using a Bio-Rad Gene Pulser XCell (Bio-Rad Laboratories, Hertfordshire, UK) at 320 V/500 μ F with 20 μ g plasmid DNA/0.4-cm electroporation cuvette. Before stimulation, appropriately transfected or untransfected cells were incubated in serum-free medium (DMEM supplemented with 2 mM glutamine, appropriate antibiotics, 50 μ g/ml hygromycin, and 10 mM HEPES) for 16 h for Western blotting for pERK1/2, pJNK1, and pP38 and cAMP assays or medium containing 10% serum for 24 h for sulforhodamine B staining. Serum deprivation for 16 h is common practice in cell signaling studies (16, 17, 68, 69). Nevertheless, the time-dependent effects of starvation on basal MAPK phosphorylation were first evaluated (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). Agonist stimulations were performed at 37 C in either serum-free medium or medium containing 10% serum after appropriate incubation with chemical inhibitors as described in the figure legends. HEK293 cells stably expressing the rat GnRH receptor (designated SCL60) generated previously within our laboratory, L β T2 cells, and CHO- M_2 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, appropriate antibiotics, and (for SCL60 and CHO- M_2 cells) 500 μ g/ml geneticin (G418) (PAA Laboratories, Somerset, UK).

Preparation of Cellular Extract and Immunoblotting

After ligand stimulation, cell monolayers were placed on ice, washed once with ice-cold Dulbecco's PBS, and lysed in a Nonidet P-40 solubilization buffer (250 mM NaCl, 50 mM HEPES, 0.5% Nonidet P-40, 10% glycerol, 2 mM EDTA, pH 8.0) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin. Solubilized lysates were clarified by centrifugation at 20,000 \times g for 15 min, and nuclear contents were sheared by subsequent sonication. Sample protein concentrations were measured using the modified Bradford assay (Bio-Rad) and diluted to a concentration of 1 mg/ml total protein. Clarified whole-cell lysates were mixed with an equal volume of 2 \times Laemmli sample buffer and resolved by SDS-PAGE. After electrophoretic separation, proteins were electroblotted on to polyvinylidene difluoride membranes (NEN Life Sciences, Buckinghamshire, UK) for protein immunoblotting. Polyvinylidene difluoride membranes were blocked in a 4% BSA (50 mM Tris-HCl, 0.05% Tween 20, 0.05% Nonidet P-40, pH 7.0) blocking solution. Immunoblotting of endogenous pERK1/2, pJNK1, or pP38 was performed using a 1:1000 dilution of rabbit antihuman phosphorylated ERK1/2, JNK1, or P38 antiserum (Cell Signaling, Hertfordshire, UK), respectively. A 1:1000 dilution of rabbit antihuman total ERK1/2 antiserum was used to verify protein loading. Visualization of the phosphorylated or unphosphorylated protein was achieved by addition of a 1:10,000 dilution of alkaline phosphatase-conjugated polyclonal antirabbit IgG as a secondary antibody. Each alkaline phosphatase-labeled protein was visualized using an enzyme-linked chemifluorescence reaction (Amersham) and quantified using a Typhoon 9400 PhosphorImager GE Healthcare.

Phosphoinositide Hydrolysis

Assays for ligand stimulation of inositol phosphate production were performed as previously described (66, 67). Briefly, appropriately transfected MEF S19 cells were labeled overnight with 1 μ Ci/ml myo-D-[3 H]inositol (Amersham) in inositol-free DMEM containing 1% dialyzed fetal calf serum before being incubated in 0.5 ml buffer A (140 mM NaCl, 20 mM HEPES, 8 mM glucose, 4 mM KCl, 1 mM MgCl $_2$, 1 mM CaCl $_2$, 1 mg/ml BSA) containing 10 mM LiCl at 37 C for 30 min. Thereafter, appropriate ligand stimulation was carried out

for an additional 30 min. The reaction was terminated by removal of the medium and addition of 0.5 ml of 10 mM formic acid. The [^3H]inositol phosphates were isolated from the formic acid extracts using Dowex AG 1-X8 ion exchange resin (Bio-Rad), collected with 1 M ammonium formate containing 0.1 M formic acid and quantified by liquid scintillation counting.

Membrane Preparation

Cells were collected in a harvesting buffer (20 mM HEPES, 100 mM EDTA, pH 7.5) and ruptured with 20 strokes of a glass dounce homogenizer. Nuclei and unbroken cells were separated by centrifugation at $200 \times g$ for 15 min. The resultant supernatant was then subjected to a high-speed spin at $40,000 \times g$ for 45 min and resuspended in a GTP γS assay buffer (5 mM MgCl_2 , 100 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol). To ensure optimal assay conditions, membranes were prepared fresh rather than stored. Membrane concentrations were determined as previously described (70).

Scintillation Proximity Assay

The SPA was performed as previously described (71). Briefly, cell membranes expressing the receptor of interest (approximately 75 μg protein/well) were incubated in the presence or absence of ligand and 200 pM [^{35}S]GTP γS for 1 h at 25 C. After incubation, membranes were solubilized in a 0.3% Nonidet P-40 solution for 30 min. Thereafter, antibodies (using dilutions of 1:440 for $\text{G}\alpha_{i1/2}$ and $\text{G}\alpha_{i3}$ and 1:1000 for $\text{G}\alpha_{q/11}$) and anti-IgG-coated SPA beads were added and incubated for an additional 3 h. Plates were centrifuged at $3000 \times g$ for 10 min and counted on a Wallac MicroBeta Trilux β -counter [PerkinElmer Life and Analytical Sciences, (UK) Ltd., Buckinghamshire, UK].

Measurement of Intracellular cAMP

After a 30-min incubation with 1 mM 3-isobutyl-1-methylxanthine and appropriate ligand stimulation, cell monolayers were placed on ice, washed twice with ice-cold PBS, and lysed in 0.1 M HCl. Intracellular cAMP concentrations were determined using an ELISA kit (Biomol, Exeter, UK) as per the manufacturer's instructions.

Measurement of Cell Growth

After continuous agonist stimulation, cell monolayers were placed on ice, and an equal volume of cold 25% trichloroacetic acid was added directly to the culture medium. Cells were left at 4 C for 1 h after which cell growth was determined using the sulforhodamine B assay as previously described (72).

Statistical Analysis

All experiments were repeated independently at least three times. In addition, all assays were performed in triplicate. Statistical significance was set at $P < 0.05$, indicated by asterisks in figures, and analyses were performed using the Student's t test. For agonist dose-response analyses, data representing the mean \pm SE from at least three independent experiments were plotted and analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Sigmoidal dose-response curves were fitted to the relevant data sets and the EC_{50} value determined.

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Antiproliferative Effects of GnRH Agonists: Prospects and Problems for Cancer Therapy

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Key Words

Cancer therapy • Cell proliferation •
GnRH agonists • GnRH receptors • Reproduction

Abstract

Gonadotropin-releasing hormone (GnRH) receptor activation has been demonstrated to inhibit cell proliferation in vitro and in vivo. These effects are dependent on the degree of receptor expression and the intracellular signaling protein milieu. The physiological and pathophysiological relevance is largely undefined, and its potential for exploitation in the treatment of specific malignancies is the subject of ongoing investigations. GnRH receptors are expressed in embryonic, juvenile and adult tissues, including brain, pituitary, gonads, accessory reproductive organs and placenta. The levels of receptor expression vary, from high in pituitary gonadotropes to low in peripheral tissues, although quantification of functional receptor protein has been determined in relatively few cell types. Roles for GnRH receptor signaling at different stages of animal development and its influence on reproductive health remain largely unexplored, except in cases of hereditary hypogonadal infertility. In addition to regulating hormone secretion, GnRH is postulated to act as a chemokine or a growth- and differentiation-inducing fac-

tor. Hence, receptor activation may influence the function of neuronal networks in the brain and the maturation of reproductive tissue epithelia. GnRH may also potentially influence the biology of cancerous cells in reproductive tissue since receptor activation may signal terminal differentiation, cell cycle arrest or apoptosis. In this context, the cell surface expression of GnRH receptor is important since it influences the intensity of intracellular signaling, and correlates with the ability to inhibit proliferation in transformed cells in vitro. Here, we review data on the effects of GnRH agonists on cell proliferation and apoptosis, and put forward hypotheses for investigation to determine whether the GnRH receptor acts as a tumor suppressor in neuroendocrine or epithelial cells.

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Introduction

Gonadotropin-releasing hormone (GnRH) is a ten-amino-acid residue neuropeptide hormone (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) involved in the control of the reproductive system [1]. Pulsatile secretion of GnRH from hypothalamic neurons acts upon receptors in the anterior pituitary to stimulate release of the

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gonadotropins: luteinizing hormone and follicle-stimulating hormone. In mammals there are two forms of GnRH: GnRH-I which regulates gonadotropin production and GnRH-II which is extrahypothalamic and appears to have a neuromodulatory role in influencing reproductive behavior [1]. The GnRH-II precursor gene is inactive or deleted in certain mammalian species [2]. Similarly, the cognate receptor for GnRH-II (the type II GnRH receptor) is disrupted by frameshift mutations or premature stop codons [2–4]. Thus it appears that endogenous GnRH-II signaling occurs through the type I GnRH receptor in relevant mammalian species, including man.

Clinically, GnRH analogs (synthetic agonists and antagonists) are used to influence ovulation in assisted reproduction and for the treatment of a wide range of hormone-dependent pathologies [5]. Chronic stimulation of pituitary GnRH receptors by exogenously administered GnRH analogs leads to decreased luteinizing hormone and follicle-stimulating hormone production. This results in a decline in circulating sex steroid hormone levels, leading to pharmacological castration. Nonetheless, the occurrence of GnRH and GnRH receptors in extrapituitary tissues such as ovary, endometrium, placenta, breast, testis and prostate suggests that GnRH analogs may also have direct therapeutic actions at these sites [6–8]. Here, we review evidence concerning the distribution and temporal variation in expression of GnRH and GnRH receptors, the potential function of this expression and its possible relationship to a selection of malignancies. We also reconsider the utilization of GnRH agonists in the treatment of reproductive tissue malignancy and present an updated perspective on this field of research. In order to retain focus, we do not review the application of GnRH antagonists or cytotoxic GnRH analogs.

GnRH Receptor Expression: Tissue Distribution and Developmental Modulation

The cDNAs and genes encoding human, mouse and rat GnRH precursors and GnRH receptors were characterized between 1984 and the mid-1990s [9–11]. Early evidence suggested that these genes were expressed in hypothalamus, pituitary, gonads and the reproductive tract. Since Northern blot signals were relatively weak, these data suggested that GnRH gene expression occurs in a small population of cells in discrete tissues. Similar results from numerous reverse transcription polymerase chain reaction analyses and *in situ* hybridization and im-

munohistochemistry studies confirm this interpretation. However, the full spectrum of mammalian cell types expressing functionally important levels of GnRH system components in adult, juvenile or embryonic tissues currently remains uncertain.

Epithelial cells in adult reproductive tissues have been the focus of investigation as possible sites for autocrine/paracrine effects of GnRH [12–15], although additional expression of GnRH components in the neuronal compartment within the reproductive system has not been explored in detail. A small number of gene promoter-reporter experiments [16, 17], some elegantly performed in transgenic mice [18], have not fully addressed the possibility that autocrine/paracrine GnRH systems exist in these tissues. Perhaps cell-targeted transgenic GnRH receptor expression would be useful in providing data regarding the functional consequences of GnRH system upregulation in particular tissues. For the time being, analyses of the developmental origins of reproductive tissue epithelia suggest that certain cell lineage similarities are consistent with GnRH system expression in quite diverse types of epithelia. For example, pituitary gonadotropes develop from oral ectoderm epithelial cells and mammary gland epithelial cells develop from epidermal ectoderm. Also, ovarian surface epithelial cells and testicular Sertoli cells develop from celomic epithelium whilst prostate epithelial cells develop from urogenital sinus endoderm (fig. 1). These cell lineages probably share aspects of gene programming given that overlapping clusters of transcription factors are likely to be active in each lineage at particular periods during development. Furthermore, there is evidence that the GnRH system may be active at very early stages of development [19] and is then subject to developmental repression. However, more knowledge on the properties of the gene promoters of the GnRH system is required to understand where and when tissue-specific GnRH gene expression normally occurs. The availability of transgenic mice with the GnRH-I proximal promoter (–3.4 kb) driving green fluorescent protein is valuable for such studies [20–22].

The proximal promoters for prepro-GnRH-I and the type I GnRH receptor genes both possess multiple DNA sequence elements likely to influence cell type-specific expression. We analyzed the proximal 4-kb DNA sequences in the 5' flanking region of each gene for putative transcription factor binding sites using bioinformatics tools: Ensembl database (<http://www.ensembl.org>) and Genomatix MatInspector software (<http://www.genomatix.de>; fig. 2). Certain transcription factor binding sites consistent with epithelial expression are present in

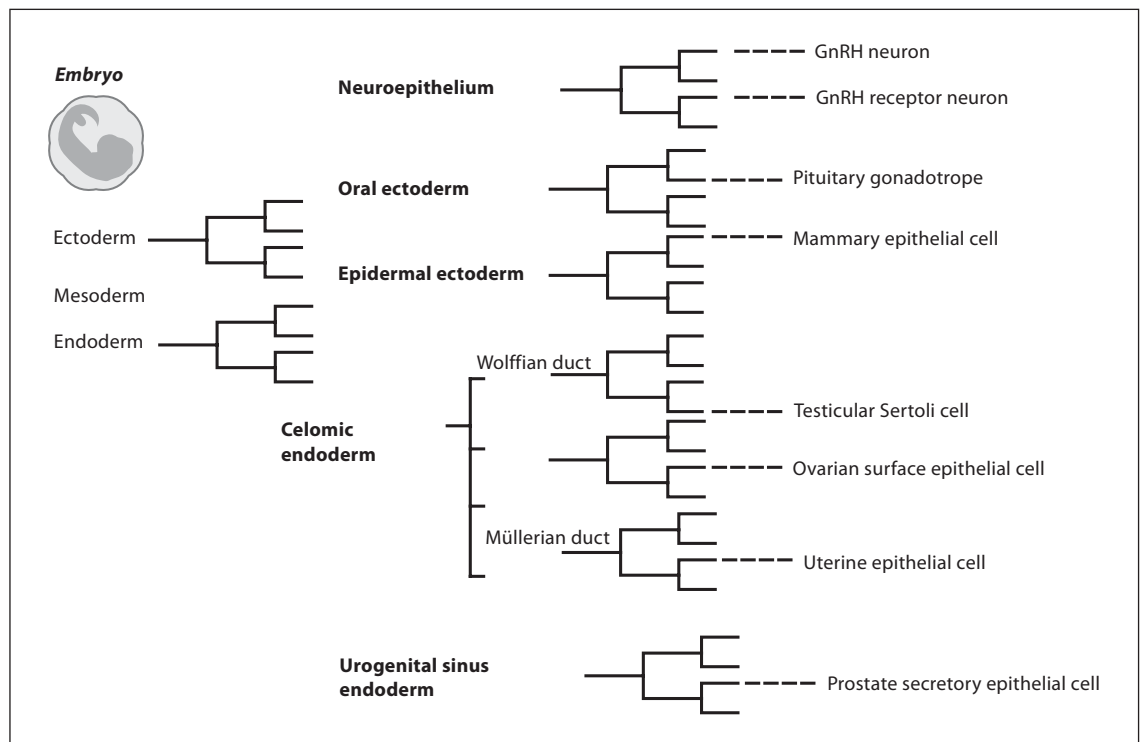


Fig. 1. Selected cell lineage relationships in reproductive tissues. The GnRH system is active during early stages of development and cells retaining expression of GnRH system components in the brain, pituitary and reproductive tissue epithelia have divergent developmental origins. Transcription factors responsible for determining tissue-specific GnRH receptor expression in relation to cell lineage have not yet been fully identified.

both human and rodent genes. For example, pituitary cell lineages and prostate basal epithelial cells both express the homeobox domain transcription factor NKX3.1 [23] and putative binding sites for this protein occur in the proximal promoters of GnRH system genes. Interestingly, NKX3.1 expression is extinguished in mature gonadotropes and in mature secretory prostate epithelial cells, but is expressed in immature cells [24], suggesting a potential transient role for GnRH signaling during cell maturation. The GnRH system gene promoters also possess potential binding sites for the transcription factor Nanog, which is characteristically expressed in pluripotent epithelial stem cells [25]. However, the complexity and relative importance of *cis*-interactions at the proximal promoters (including effects mediated by the distal promoter region and potential enhancer or repressor elements) cannot be understood without experimental investigation. Hence, these speculative *in silico* analyses of the GnRH precursor and GnRH receptor proximal promoters suggest important regulatory mechanisms wor-

thy of further investigation. Investigation of physiological or pathophysiological mechanisms responsible for inducing elevated GnRH receptor expression, including the identity of key transcription factors, would result in useful contributions to understanding the significance of GnRH system activity in diseases affecting reproductive tissues and in developing interventions for clinical therapy.

Potential Functions of GnRH Receptors in Different Tissues

GnRH receptor activation elicits regulated secretion from pituitary gonadotropes. It seems plausible that the receptor may serve similar roles in other reproductive tissues, such as secretory epithelium. Additional roles may involve the maintenance of cell differentiation in adult tissues or determination of the organization of cells in developing tissues (fig. 3). These putative cellular pro-

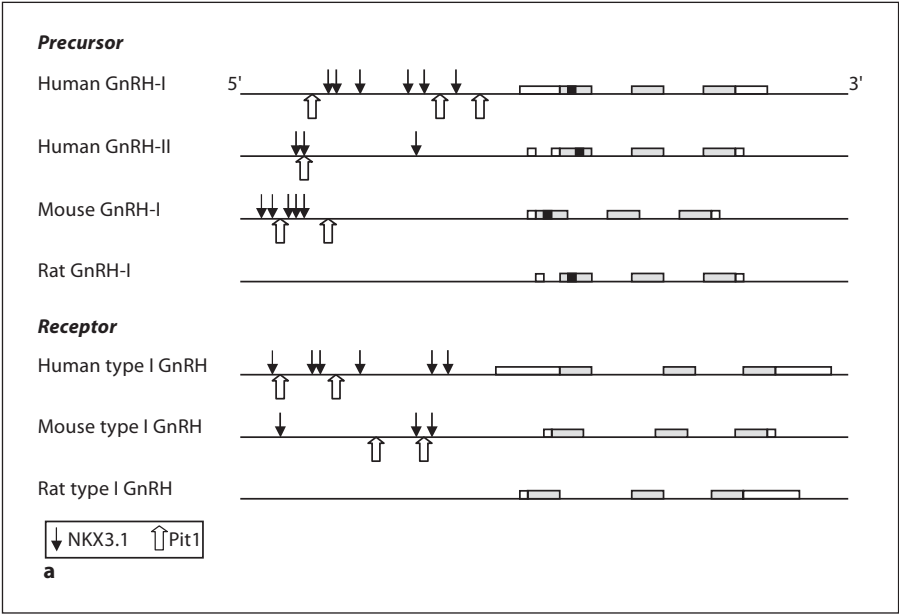


Fig. 2. a Organization of genes encoding prepro-GnRH-I (and prepro-GnRH-II in humans) and type I GnRH receptors in man and laboratory rodents. Rectangular boxes represent exons. Coding regions are shaded, with GnRH peptide denoted by black shading. Selected putative transcription factor-binding sites identified by bioinformatics analyses are arrowed and the comparative abundance of individual binding sites between species is listed. **b** Putative binding sites for Nanog in proximal promoter regions (first 4 kb analyzed for each gene) suggest possible expression of the GnRH system in cells expressing this transcription factor (often associated with pluripotent stem cells). Note the occurrence of binding sites for prostate epithelial marker NKX3.1 and for pituitary markers Pit, LHX3 and LIM known to be important in regulating GnRH system gene expression.

	Transcription factor site	Human GnRH-I	Human GnRH-II	Mouse GnRH-I	Rat GnRH-I
Precursor	Pit1	3	1	2	2
	Pitx1	1	2	2	1
	LHX3	3	3	2	2
	LIM	8	0	1	3
	NKX3.1	6	3	4	3
	Nanog	5	2	2	1
	SF-1	2	1	0	1
	CREB	2	1	3	2
	Ets	3	5	1	1
	Abl	2	2	2	0
	Transcription factor site	Human type I GnRH	Mouse type I GnRH	Rat type I GnRH	
Receptor	Pit1	2	2	2	
	Pitx1	1	0	0	
	LHX3	2	1	4	
	LIM	3	1	2	
	NKX3.1	6	5	2	
	Nanog	4	5	3	
	SF-1	1	4	6	
	CREB	1	1	3	
	Ets	3	1	4	
	Abl	2	0	0	

b

gramming functions may be mediated via effects on transcription factor activity following GnRH receptor activation [26, 27]. However, it remains uncertain whether expression of GnRH receptor in ovarian surface epi-

thelium, mammary gland epithelium, uterine epithelium or prostate epithelium serves a physiologically important role or whether it is the result of aberrant gene expression due to disease pathology.

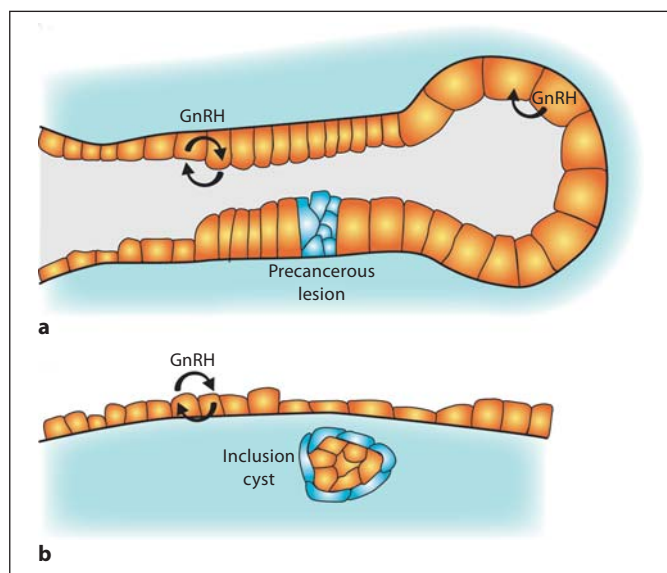


Fig. 3. Hypothetical sites for GnRH agonist action in normal or diseased secretory glands (**a**) or surface epithelium (**b**) found in reproductive tissues (such as breast, prostate, uterus and ovary). Glandular epithelium or surface epithelium both contain a variety of epithelial cell subtypes, some of which may express an autocrine/paracrine GnRH system, whilst other cells (in precancerous lesions or inclusion cysts) may aberrantly express elevated levels of GnRH receptor and would, therefore, be targets for therapeutic application of GnRH agonists.

Relationship of GnRH Receptor Expression to Oncogenesis

The expression of GnRH receptors has been reported in a variety of tumor-derived samples and its functional effects have been studied in animal models of tumor growth. Several groups reported that mRNA coding for the receptor is expressed in prostate [28–30], breast [31–33], endometrial [34, 35] and ovarian [36, 37] cancers – GnRH receptor expression has also been reported in nonreproductive tissue malignancies [38–41] – and the nucleotide sequence of the cDNA coding for the type I GnRH receptor in breast and ovarian tumors is identical with the receptor present in the pituitary [31]. In tumor-derived cells of the male reproductive tract, receptor expression has been detected at the protein level. Western blot analysis using a monoclonal antibody raised against the native human pituitary receptor [42] allowed Limonta et al. [43] to demonstrate a protein of approximately 64 kDa in two prostate cancer cell lines. Taken together, these data indicate GnRH receptor expression in a range of reproductive tissue malignancies, although the func-

tionality of the receptor and the potential downstream effects of its activation remain contentious. Traditionally, receptor expression within samples has been characterized in terms of radioligand binding properties (receptor abundance and ligand binding affinity); however, various controversial results have thus far been published. Some studies have revealed only binding sites which exhibit low affinity for GnRH-I analogs in prostate cancer cells [44] and others have observed similar binding parameters in endometrial and ovarian cancer cells and tissues [34, 45]. Other studies have reported the presence of two types of receptors, one with high and one with low binding affinity in breast and gynecological cancers [46–48]. In contrast, one single class of high-affinity binding sites has been reported in breast, ovarian and endometrial cancers by different groups [49–51]. At present, questions concerning the binding affinity of these receptors in tumor-derived samples and transformed cell lines are still a matter of debate, and ones which must be addressed before significant advances can be made.

The presence of both GnRH and type I GnRH receptors in tumors of the reproductive tract nevertheless prompted investigations into the possible role(s) of GnRH in the local control of tumor growth [52]. Research in the laboratory of Motta and coworkers [44] has shown that GnRH agonists exert an inhibitory effect on the proliferation of prostate cancer cells in a dose-dependent manner, and much of the work could be elegantly verified when the cells were xenografted into nude mice [53]. Others have confirmed similar antiproliferative activities of GnRH agonists when applied to breast, ovarian and endometrial cancer cells, and we have developed receptor-expressing model cell lines for use both in vitro and in vivo [6, 54–56]. Since activation of the receptor in tumor cells is associated with antiproliferative effects, it is possible to hypothesize that in malignancies of the reproductive tract any locally expressed GnRH system might act as a negative regulator of tumor growth. Furthermore, this suggests that GnRH autocrine/paracrine signaling is often defective within growing tumors, possibly due to inadequate local production of GnRH.

GnRH Analogs in Clinical Trials for Cancer Therapy

GnRH agonists have been used in prostate cancer clinical trials since the early 1980s [57] and in uterine leiomyoma trials since the early 1990s [58]. Studies of GnRH agonist effects in recurrent uterine endometrial cancer, premenopausal breast cancer and ovarian cancer have been

conducted [59–62]. In all of these trials the main aim of GnRH agonist treatment was the suppression of sex steroid hormone levels to limit growth of steroid-responsive malignant cells. A variable proportion of tumors exhibit objective growth inhibition attributable to GnRH agonist treatment. Correlations between levels of GnRH receptor expression and disease outcome have occasionally been reported [63] and suggest a direct effect of GnRH agonists on tumor cells, including effects on steroid-insensitive cells. However, evidence for direct effects on tumor cells in patients needs to be verified by rigorously controlled trials with appropriate sample sizes. Furthermore, understanding how GnRH receptor activation may affect cancer lesion progression is an important problem concerning the patient-specific (or even prophylactic) use of GnRH agonists in the treatment of malignancy. For example, does GnRH receptor signaling maintain the differentiation state of normal cells, alter their mobility or induce apoptosis? Additionally, are any of these processes altered in transformed cells exposed to GnRH agonists? Information derived from basic science investigations may influence the way in which clinical trials are designed in the future.

Details regarding the mechanism of GnRH agonist-mediated inhibition of cell growth are only beginning to emerge. Some groups [64, 65] have suggested that activation of the receptor in peripheral malignancies results in activation of the G protein $G\alpha_i$ and that the consequent decrease of intracellular 3',5'-cyclic adenosine-5'-monophosphate results in activation of phosphotyrosine phosphatases, thus interfering with growth factor-induced tyrosine phosphorylation and downstream intracellular signaling. Others [66] have implicated the presence of a second type of GnRH receptor, with a preference for GnRH-II, in ovarian and endometrial cancers, which transmits significantly stronger antiproliferative effects than the type I receptor. Interestingly, neither we nor Grosse et al. [67] found any evidence of $G\alpha_i$ coupling using direct analytical techniques. In addition, we have shown that, in man, the type II receptor is effectively silenced by a premature stop codon and a frameshift deletion [2]. Clearly, further elucidation of the complex signaling cascades activated in response to GnRH agonists is necessary before we can begin to understand the mechanism of GnRH agonist-mediated cell growth inhibition. Also, the use of cultured cells currently forms the basis of experiments investigating GnRH agonist-mediated cell growth inhibition. The relevance of these models to in vivo disease pathology needs to be addressed and data generated using cell lines should be carefully characterized and compared to primary tumor samples.

Developing Models for Characterization of GnRH Agonist-Mediated Cell Growth Inhibition

Several models need to be developed to explore the role of GnRH receptor signaling in reproductive tissue malignancy. The different models are needed to encompass mechanisms particular to prostate cancer, ovarian cancer, uterine cancer and breast cancer since cell type-specific signaling has been proposed to be an important factor influencing responses to GnRH [68]. Alternative types of cultured human cells derived from each of these tissues are available, including cells immortalized by experimental manipulations [69] rather than by acquisition of disease-associated oncogenic mutations. More recently, pluripotent stem cells which may be the precursors of certain types of carcinoma have become accessible for in vitro studies [70]. Analyses using these cells should more closely reflect the physiological responses elicited by GnRH in early-stage disease rather than in well-progressed metastatic cells.

The determination of precisely how GnRH receptor activation leads to cell cycle arrest and the induction of apoptosis remains a challenge. A sufficient level of cell surface GnRH receptor expression is required to elicit growth inhibition [71, 72]. The mechanisms regulating the routing of GnRH receptor to the cell surface are poorly understood [73], although the process appears to be tightly regulated since often only a small proportion of total receptor protein resides in the plasma membrane in stably transfected cells. The ability to pharmacologically modulate GnRH receptor trafficking to the plasma membrane is an attractive therapeutic goal and the increased surface expression of wild-type as well as mutant receptors using cell-permeable small molecules demonstrated that this is achievable [74]. Once at the cell surface, the type I GnRH receptor associates with protein scaffolds which facilitate signaling, the details of which are beginning to be characterized in depth [75]. Further studies should determine whether cell growth inhibition is induced by signaling from the receptor directly to the machinery regulating apoptosis or whether there is an indirect effect through de novo gene expression or through compromise of the mitotic apparatus (such as checkpoint control, spindle dynamics and altered cell adhesion during cytokinesis; fig. 4, 5).

Signaling Downstream from the GnRH Receptor

Theoretically, GnRH agonist-mediated inhibition of cell growth may involve activation of a number of intracellular pathways. Binding of agonists to the GnRH re-

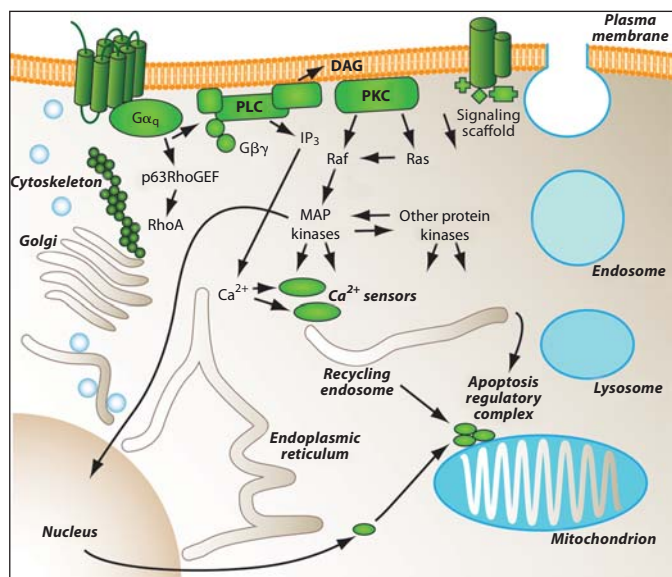


Fig. 4. Multiple signaling routes from the cell surface GnRH receptor to the machinery regulating the intrinsic pathway of apoptosis at the mitochondrial membrane. Signaling diverges following activation of Gα_{q/11} and Gβγ, leading to activation of PLCβ, generation of IP₃, elevation of intracellular Ca²⁺, activation of DAG-sensitive proteins, such as PKC, and activation of transcription factors. Apoptosis occurs in response to ‘inappropriate’ signaling, but commitment to cell death may be modulated by a range of factors associated with, for example, Ca²⁺ sensors and cytoskeletal components, including the focal adhesion complex and actin fibers.

ceptor triggers activation of Gα_{q/11} proteins, which activate phospholipase C (PLC) isoforms β1–β4 to stimulate turnover of inositol trisphosphate (IP₃) and diacylglycerol (DAG). These signaling molecules lead to the release of Ca²⁺ from intracellular stores and protein kinase C (PKC) activation, respectively. Activation of Gα_{q/11} also releases the Gβγ dimer which can independently activate several isoforms of PLC, including PLCβ₂, PLCβ₃ and PLCε [76, 77]. Other downstream effects of Gα_{q/11} include interaction with RhoA via the Dbl family guanine nucleotide exchange factors (GEF) such as p63RhoGEF, Trio and Kalirin [78]. The antiproliferative response provoked by GnRH agonists may, therefore, be mediated by intracellular Ca²⁺ elevation, downstream effects of DAG or small GTPase activation (fig. 4).

The cellular response to elevated intracellular Ca²⁺ varies according to differentiation state. Activation of pituitary gonadotrope GnRH receptors results in oscillations in the level of intracellular Ca²⁺ linked to activation of regulated exocytosis [79–81]. This process requires the

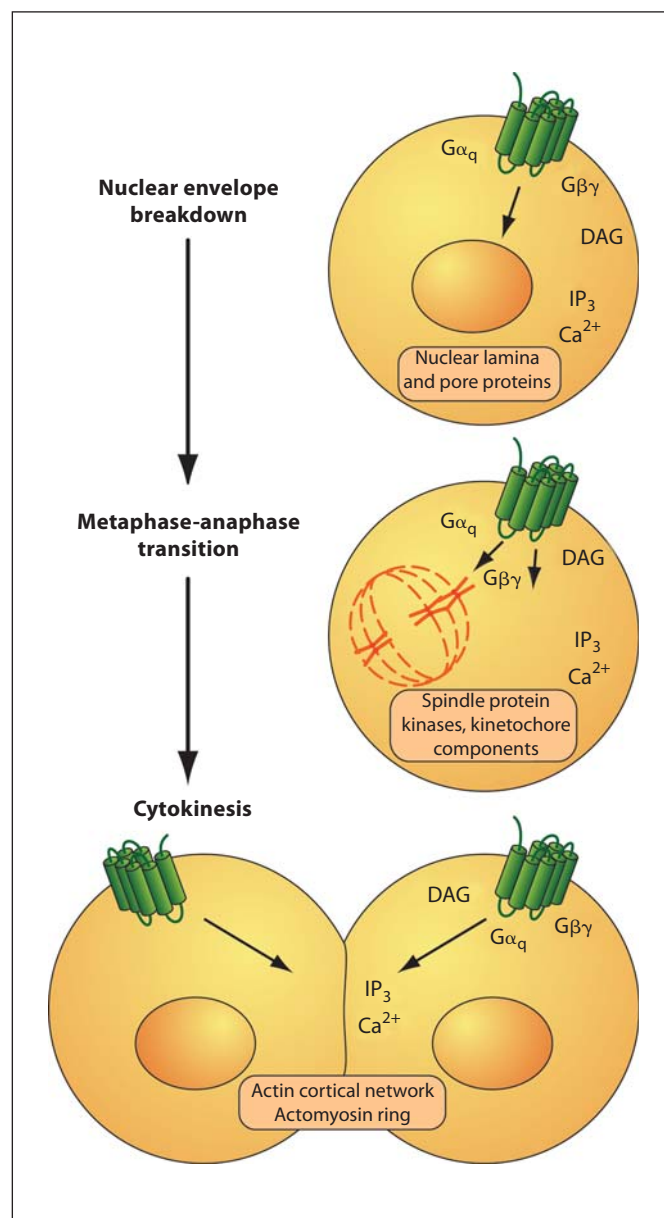


Fig. 5. Selected phases of mitosis potentially sensitive to aberrant GnRH receptor signaling. Inappropriate elevation of intracellular Ca²⁺ or protein phosphorylation following exposure to GnRH analogs may disrupt processes such as nuclear membrane disassembly, spindle function or cell separation. Nuclear membrane disassembly requires tightly regulated modifications of proteins forming the nuclear membrane lamina and pores. Correct segregation of replicated chromosomes requires coordinated regulation of spindle protein kinases and kinetochore components. Separation of daughter cells requires regulation of proteins forming the cortical cytoskeleton in the region of the actomyosin ring. Disruption of some of these processes, by induction of GnRH receptor signaling, may lead to stage-specific arrest of mitosis followed by apoptosis as a consequence of inappropriate elevation of intracellular Ca²⁺, small GTPase activity, or alterations in DAG-activated PKC isoforms.

Table 1. Transcription factors, enzymes and receptors modulated following activation of ERK1/2, one of the principal protein kinases activated by GnRH receptor agonists

Protein targets downstream from ERK	Function: cellular response	References
Brf1	Transcription factor subunit: hypertrophy	107
Calpain	Protease: cell cycle progression, mobility, long-term potentiation, cell fusion	108
Creb	Transcription factor: cancer	109
EGFR	Receptor: phosphorylation by ERK blocks EGFR signaling	110
Elk1	Transcription factor: transcription of c-Fos and PARP1, growth, proliferation, differentiation	111, 112
ER81	Transcription factor: gene expression	113
c-Fos	Forms AP-1 transcription factor with c-Jun: proliferation, differentiation, response to stress	114
IKK α	Kinase: activation of transcription factor NF- κ B	115
MLCK	Kinase: cytoskeletal reorganization	116
MKP	Phosphatase: regulation of MAPKs	117
MSK1/2	Kinase: phosphorylation of histone H3, HM.GI4 and Atf1	118
c-Myc	Transcription factor: cell cycle regulation	119
p53	Transcription factor: cell cycle regulation, tumor suppressor	120
p90Rsk	Kinase: phosphorylation of transcriptional regulators and polyribosomal proteins, cell cycle regulation	121
Pax6	Transcription factor: development	122
PEA3	Transcription factor: oncogenesis	123
PO-B	Transcription factor: differentiation	124
Sap1/2	Transcription factor: gene expression	125
Sos	Guanine nucleotide exchange factor: required for efficient Ras signaling, development	126
Ubf	Transcription factor: ribosomal transcription	127

expression of a specialized apparatus, including an array of plasma membrane ion channels, to ensure a return to homeostasis following cellular activation. It is possible that activation of GnRH receptors expressed in inappropriately differentiated cells may lead to a Ca^{2+} -mediated stress response [82]. However, antiproliferative effects caused by transient increases in Ca^{2+} may also be influenced by Ca^{2+} -sensing proteins such as the neuronal calcium sensor (NCS) family or the calmodulin family.

NCS proteins are small cytoplasmic proteins able to associate with membrane phospholipids and a variety of integral membrane proteins. They can modulate IP_3 signaling, regulate exocytosis and influence G protein-coupled receptor activity [83]. Changes in protein conformation occur following the binding of Ca^{2+} , thus mediating the response to receptor activation [83, 84]. A role for NCS proteins in modulating antiproliferative effects is supported by a number of studies [85–87]. One NCS protein, recoverin, is expressed in a number of tumor types including breast cancer, ovarian cancer and endothelial carcinomas [86], some of which have been shown to respond to GnRH analogs. Another NCS, visinin-like protein-1, is highly expressed in less aggressive squamous cell carcinomas, whereas little or no expression is ob-

served in invasive squamous cell carcinomas [88]. Visinin-like protein-1 influences cell adhesion and migration through regulation of fibronectin receptor expression [89]. Therefore, loss of expression or inhibition of this Ca^{2+} sensor may contribute to tumor progression and metastasis. Ca^{2+} binding by other proteins has been shown to inhibit apoptosis and promote cancer cell survival. These include calmodulin, which promotes cell survival via activation of calcium/calmodulin-dependent kinase 2 [90] and by modulation of ERK1/2 activity [91] (see below). Whether GnRH agonists induce protracted elevation of intracellular Ca^{2+} in malignant cells, and how this impinges on Ca^{2+} -sensing proteins, has not been studied in detail.

In addition to IP_3 -stimulated Ca^{2+} release, PLC activity also generates DAG, which is a potent activator of PKC isoforms and other target proteins such as β -chimerin [92, 93]. PKC isoforms remain activated after the original activation signal or the Ca^{2+} transient has waned [94]. PKC α triggers a protein kinase cascade, consisting of Raf, mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) and ERKs 1 and 2. PKC also activates Raf indirectly through activation of Ras [95]. Ras, Raf, MEK and ERKs 1 and 2 are ubiquitously expressed

in mammalian cells and are implicated in control of cell proliferation, differentiation, survival and transformation [96–98]. Activated ERKs translocate to the nucleus where they phosphorylate transcription factors as a means of regulating gene expression [99] (table 1). ERK has been implicated in cell cycle arrest in a number of cell types [100, 101], and a large body of evidence indicates that the strength and duration of ERK activation is critical in determining the cellular response. Transient or cyclic ERK activation has been linked to progression of the cell cycle, whilst sustained activity can lead to growth arrest and differentiation [102–104]. Thus the spatial and temporal activation of ERK is kernel in determining cellular responses such as survival, proliferation and cell cycle arrest. A variety of processes influence the nature of the ERK response. Significantly, differences in receptor levels markedly affect the intensity and duration of ERK activation [103].

Very recently it has been shown that $G\alpha_{q/11}$ can activate RhoA via p63RhoGEF, Trio and Kalirin [78] and, for this reason, potential activation of RhoA in response to GnRH has yet to be examined. Overexpression of RhoA has been shown to induce growth arrest in several cell types by disrupting the actin cytoskeleton and microtubules [105]. Cytoskeletal rearrangement occurs in cells treated with GnRH [106; for references 107–127 see table 1], although it is not known whether this is due to RhoA-mediated effects and requires further investigation.

In summary, a number of GnRH agonist-stimulated signaling cascades, one example being the ERK pathway, have been implicated as being causative in antiproliferation and apoptosis. Conversely, multiple signaling cascades activated following GnRH receptor activation, some of which we have highlighted here, require further investigation.

Conclusions and Future Perspectives

The GnRH system has been the subject of intensive research for almost 40 years and a role for GnRH agonist signaling in the inhibition of reproductive tissue malignancy has been promulgated for more than 20 years. Reproductive biology, peptide chemistry, experimental pharmacology, molecular and cellular biology and extensive clinical studies have all been brought to bear on the therapeutic development of GnRH analog drugs. The discovery that GnRH receptor activation can inhibit the growth of tumorigenic cells (by direct or indirect action)

continues to offer therapeutic potential. However, more research is required to determine the extent to which the antiproliferative effects of GnRH agonists demonstrated in the laboratory are translatable into the treatment of human malignancies. Although the main therapeutic effect of GnRH agonist therapy is due to sex hormone deprivation, it remains possible that particular subgroups of tumor cell types are prone to express elevated levels of GnRH receptor which may be a target for direct action. Therefore, more screening and phenotyping may identify patients likely to possess sufficiently high levels of GnRH receptor to ensure a significant additional response to GnRH agonist therapy. The data from such analyses may help to refocus clinical studies. For example, prospective studies involving classification of patients according to the level of GnRH receptor expression in malignant tissue have not been attempted so far. Advances in understanding where and when GnRH action is important in disease pathology, how levels of receptor expression can be manipulated pharmacologically and how receptor activation ultimately leads to cell apoptosis represent important goals in realizing the potential of a rare phenomenon – a G protein-coupled receptor linked to cell growth inhibition with sufficiently restricted tissue distribution to offer therapeutic advantages and manageable or reversible side effects.

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